PHD

In vitro morphogenic studies in Arabidopsis thaliana (L.) Heynh

Taylor, Nigel James

Award date:
1991

Awarding institution:
University of Bath

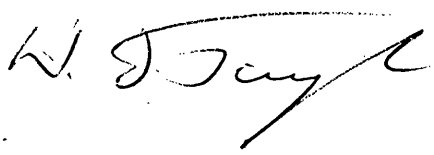
IN VITRO MORPHOGENIC STUDIES IN ARABIDOPSIS THALIANA (L.) Heynh

Submitted by Nigel James Taylor BSc.

for the degree of Doctor of Philosophy

of the University of Bath

1991



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

I would like to thank Professor Henshaw for his guidance and considerable input into this thesis at its critical times. With his help I hope that it has been lifted above the mundane.

I must also mention Chris Davey for his advice in all things technical andd mechanical; especially hubs, headsets and bottom brackets, as well as much useful discussion concerning the effect of venue in determining the degree of bounce required.

Similar thanks must also go to Pete Clark for solid anchoring during my time here, and for admirable composure under sometimes intense pressure. Must not forget all the photos too; thanks Pete.

I would also like to thank all the people that I have worked with in the lab for helping to make the bad times bearable and Vivienne for typing this so rapidly and efficiently.

Finally much credit must go to my parents for their continued support over the years although at times they must have thought it a sair fecht. Without them what follows would not have been possible.

I caught a fleeting glimpse of life,

And though the water's black as night,

The colours of Scotland make you young inside.

Runrig

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	abscisic acid
AIS	Arabidopsis Information Service
FAA	formalin/acetic acid/cheotol
hr.	hour
K	kinetin (6-furfylominopurine)
MS	Murashige and Skoog Basal medium (1962)
NAA	Napthalene - acetic acid
PAR	photosynthetically active radiation
PE	potentially embryogenic
TBA	tertiary butyl alcohol (2-Methylpropan-2-ol)
Z	zeatin (6-(4-hydroxy-3-methylbut-2-enylamino)purine)

ABSTRACT

The increasing use of Arabidopsis thaliana as a model species in plant molecular and developmental biology has made it important to design simple and efficient systems for the recovery of in vitro manipulated tissues. In addition such systems provide an opportunity to investigate the fundamental controls behind de novo morphogenesis.

A two-stage system utilising 10^{-6}M 2,4-D and 10^{-7}M BAP in the first, and 10^{-6}M BAP in the second stages was developed which was capable of inducing a caulogenic response at frequencies of between 95 and 100% from hypocotyls from seven of the nine genotypes tested. Roots were also formed at very high frequencies from this system but the two organs arose from spatially and structurally different parts of the explant.

The duration of the first-stage culture determined the type of organ and the frequency of response. While the time of maximum rhizogenic competence ocurred after four days, a minimum of six days was required to obtain competence for caulogenesis. Exposure for more than twelve days on this medium was correlated with a rapid decline in the ability to form shoots. Although both the first and second stages were manipulated, no somatic embryogenesis was observed from the cultured material.

CONTENTS

The tissue appeared to be determined for rhizogenesis at subculture to the second stage medium, but it was only competent for caulogenesis, needing an inductive exposure to the BAP medium for at least 24 hours before becoming determined for shoot formation.

The rapid, highly synchronous formation of shoots and roots after subculture to the second stage, plus the availability of a recalcitrant genotype, facilitated a detailed anatomical study into the histological basis of the organogenic competence and established that this may be related to the structure of the tissue at the time of exposure to the inductive conditions. While the roots were formed from what appeared to be preformed initials the shoots were not, while caulogenic competence was correlated with the presence of a thin layer of disorganised meristematic cells at the surface of the callus tissue. Meristematic cells were present in this form only between six and ten days in the first stage medium and were not seen at any time in the recalcitrant genotype. Continued exposure to the first stage medium produced large organised regions of meristematic tissue which appeared to be unable to undergo the correlated divisions required to form a new tunica structure.

Although no somatic embryos were recovered the presence of two globular embryoid structures seen in callus tissue cultured in the first stage medium for 20 days indicates that somatic embryogenesis may be achievable from the culture system developed in this study.

ACKNOWLEDGEMENTS

I would like to thank Professor Henshaw for his guidance and considerable input into this thesis at its critical times. With his help I hope that it has been lifted above the mundane.

I must also mention Chris Davey for his advice in all things technical andd mechanical; especially hubs, headsets and bottom brackets, as well as much useful discussion concerning the effect of venue in determining the degree of bounce required.

Similar thanks must also go to Pete Clark for solid anchoring during my time here, and for admirable composure under sometimes intense pressure. Must not forget all the photos too; thanks Pete.

I would also like to thank all the people that I have worked with in the lab for helping to make the bad times bearable and Vivienne for typing this so rapidly and efficiently.

Finally much credit must go to my parents for their continued support over the years although at times they must have thought it a sair fecht. Without them what follows would not have been possible.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The Potential of Arabidopsis thaliana as a Model Test Plant

Arabidopsis thaliana, thale cress or rock cress, is a small, annual crucifer found throughout the northern hemisphere but thought to have originated in northwestern Europe (Redei, 1975). Although it belongs to the family Brassicaceae it has no known economic value and is generally considered to be an innocuous weed. A number of A. thaliana's characteristics are, however, appreciated by plant geneticists, and since the studies of Laibach in the 1940s the value of this little "weed" has become increasingly obvious.

Traditionally, crop plants such as maize, pea and tobacco have been utilised by experimental geneticists. Unfortunately these plants are not particularly amenable to manipulation in the laboratory, being of large size with long life cycles and possessing complicated genomes. A. thaliana has many advantages over the above species that make it ideal as a model for the study of plant genetics:

1. small size. Tens of thousands can be grown to maturity in a small room (Redei, 1975) or several thousand seedlings screened in a 9.0cm petri dish (Bowman et al., 1988).

2. short generation time. Although this varies with the genotype, the most commonly used experimental types, Landsberg and Columbia, complete their life cycle in approximately six weeks (Redei, 1975; Bowman et al., 1988).

3. self fertilisation. Due to cleistogamy Arabidopsis is self fertilising with a very low outcrossing frequency of 10^{-4} (Redei, 1975). This ensures excellent homogeneity in successive generations and allows new mutants to be conserved with confidence. Cross-fertilisation can be achieved, if desired, by simple emasuclation techniques (Feenstra, 1965; Meyerowitz, 1985).

4. large seed production. Under optimum conditions each plant is capable of producing 2000-3000, or more, seeds (Laibach, 1943). This allows rapid multiplication of desired genotypes and the generation of ample experimental material.

5. small genome. A. thaliana has the smallest known genome in the plant kingdom. With an estimated genome size of 7 x 10^7 base pairs it has at least 200 times less DNA than tobacco and 800 times less than wheat (Meyerowitz, 1985).

6. non repetitive DNA. This plant has considerably less repetitive DNA than other higher angiosperms (Flavell, 1980; Bowman et al., 1988).

7. single gene expression. Meyerowitz (1987) has shown that gene expression in A. thaliana is associated with single gene sequences and not with groups of families of genes.

8. large genetic background. Scores of different wildtypes are available to the worker plus a increasing number of mutants. The latter vary from morphological to physiological and developmental and a few are now partially mapped (Haughn and Somerville, 1988).

It can be appreciated that A. thaliana is uniquely suited for laboratory use, with factors 5 to 8 making it considered by some to be the Drosophila of the plant kingdom (Conner, 1989). It has therefore attracted increasing attention in recent years and is now a major tool in the hands of the geneticists and molecular biologists.

The work in this thesis was undertaken with the intention of increasing our knowledge about the in vitro behaviour of Arabidopsis. Although a number of studies were already available it was evident that the systems in use for the recovery of plants by organogenesis and embryogenesis were not very efficient and could be improved upon. This was considered to be an important project as the reliable regeneration of whole plants from genetically transformed tissues, is central to the progress of plant genetic engineering and essential to molecular genetics contribution to crop improvement programmes. Secondly, the unique characteristics of A. thaliana listed above make it ideal as a model for the fundamental study of plant development. The amenable nature of its genome to genetic studies and the promise of future research into its gene sequences, and their relationships with whole plant development (Meyeritz and Pruitt, 1985), has the potential to reveal some of the underlying control systems behind de novo morphogenesis. No other plant has such known potential.

The work has been split into two chapters. In Chapter 1 morphogenesis is reviewed from both the practical and theoretical view points, and the current state of regeneration procedures in use for Arabidopsis is assessed. This places into context the high

frequency regeneration system developed during this study and the variables that limit it. Chapter 2 describes a detailed anatomical study of this morphogenetic system, concentrating especially on the developmental aspects of callus, root and shoot formation.

In vitro Manipulation of A. thaliana

The history of the manipulation of A. thaliana in culture dates back to 1955 and involves a number of distinct approaches. Langdridge (1955) was the first to place Arabidopsis into culture. He germinated seeds axenically and completed the whole life cycle, including reasonable seed set, on a basal medium of Knops solution, and in so doing demonstrated the responsive nature of this plant to in vitro manipulation.

During the following decade attempts were directed towards establishing and maintaining callus cultures on various media (Leowenberg, 1965; Zeibur, 1965; Shen-Miller and Sharp, 1966; Anand, 1966; Corcos and Lewis, 1971). Although Anand (1965) obtained rhizogenesis and Yokayama and Jones (1965) and Nitsch (1967) reported some indirect regeneration from seedling parts, no detailed account of caulogenesis was provided until the reports of Gresshoff and Doy (1972) and Corcos et al., (1973). In the former, haploid regenerants were recovered from anther-derived callus from three out of a total of eighteen genotypes at undisclosed frequencies, while the latter reported high-frequency (c.75%) plantlet regeneration from a one-stage system after four months on a medium supplemented with IAA and kinetin.

These two papers appear to have had considerable influence on the work of Negrutiu et al. (1975; 1976; 1978a; 1978b; 1978c) as this group continued this direction of study, basing most of their work on regeneration from long term callus cultures using basal medium similar to that of Gresshoff and Doy (1972) and relying heavily on the use of IAA and kinetin in the differentiation medium. In these papers they examined in detail, morphogenesis from callus tissue and investigated the effect of numerous variables on the regeneration frequencies.

Explants were placed on a callus inducing medium containing B5 (Gamborg, 1968) salts with 10^{-5}M 2,4-D/2.5 x 10^{-7}M kinetin and after about four weeks subcultured onto either the same medium or a callus maintenance medium also of B5 but with 5 x 10^{-6}M 2,4-D/2.5 x 10^{-7}M kinetin (Negrutiu et al., 1978a). Thereafter subculture took place every four weeks until needed for regeneration, at which time the callus was placed on the shoot induction medium. This consisted of Gresshoff and Doy basal medium (Gresshoff and Doy, 1972) supplemented with 10^{-7}M IAA and 10^{-6}M kinetin. The effect of the genotype, explant source, basal medium, growth regulator types and concentrations, subculture intervals, light and temperature effects and nitrogen additions on this system were all assessed. To summarise:

1. They found that the regeneration frequency varied with the genotype. Rarely were frequencies quoted but the genotypes could be ranked in terms of response such that Estland (57%) Coimbra Wilna Columbia (20%) Chisdra (Negrutiu, et al., 1975).

2. The explant type had a significant effect; regeneration could be achieved from any explant, but seeds and leaves were superior to stems and roots while anthers provided the most responsive tissues recording, at 67%, the highest regeneration obtained (Negrutiu, et al., 1978b).

3. B5 (Gamborg, 1968) and DBM (Gresshoff and Doy, 1972) were used as the basal media, but it later papers it was reported that MS, G5, Gresshoff and Doy, and Nitsch were all equally effective (Negrutiu et al., 1978a).

4. They found that in the first stage 2,4-D was more effective than NAA at inducing callus production and was especially so when used in conjunction with a low concentration of kinetin (Negrutiu et al., 1975).

5. The optimal growth regulator concentration for the induction of caulogenesis in the second stage was shown to be a 10:1 ratio of kinetin to IAA, but in later studies zeatin was found to be more effective than kinetin and kinetin more so that BAP (Negrutiu et al., 1978a).

6. Continuous, diffuse light was slightly better than high light levels or a photoperiod (Negrutiu and Jacobs, 1978c).

7. The standard subculture period was four weeks, but an extended period of eight weeks prior to transfer to the differentiation medium was found to improve the regeneration frequency (Negrutiu et al., 1978a).

8. A cold treatment of 4°C could rejuvenate old callus. As the callus aged it lost caulogenic potential, but a thermal shock could reverse this effect and increase regeneration by up to 200% (Negrutiu and Jacobs, 1978c).

Through their series of papers Negrutiu *et al.* demonstrated the organogenic potential of *A. thaliana* in culture, and provided a sound body of knowledge for further research. This was taken up by Scholl *et al.* (1980; 1981) who used Negrutiu's multi-step regeneration procedure to obtain haploid regenerants from anther cultures at frequencies of around 50%, and by Huang and Yeoman, who in 1984, reported somatic embryo formation from seed derived callus. Although Negrutiu (1978a) had mentioned "embryo-like structures.....in callus derived from anthers" and Scholten and Feenstra (1985) likewise, also from anther derived callus, Huang and Yeoman (1984) provide the only detailed report of somatic embrogenesis in *A. thaliana*. They found that, after an undisclosed number of passages on the callus induction and maintenance media (ones very similar to those used by Negrutiu) and transfer to the 10:1 kinetin/IAA differentiation medium, nodules were formed on the callus. These most often developed to form shoot structures, but occasionally, and especially if the auxin was removed and the cytokinin reduced, they would differentiate into small embryoidal masses. Transfer to a hormone free medium allowed the embryos to germinate and develop into plantlets. The efficiency of this system is difficult to determine as no

quantitative data is provided as to the percentage of the calluses undergoing embryogenesis or the number of embryos produced per callus.

These workers (Huang, 1985) also reported a system for the induction of somatic embryogenesis with cultures derived from late-heart-stage zygotic embryos. The latter were induced to from callus on the same first-stage medium as used for the seedling tissue but with a yeast extract supplement. The zygotic embryo derived callus was then maintained by monthly subcultures on the same medium, and the somatic embryos were detached from this tissue as they arose, and placed on a hormone free medium to allow maturation and plantlet development. Under these conditions about 10% of the cultures were capable of embryo formation, although the quality of the resulting propagules was not high. Most recently Ford (1990) has described the recovery of "structures that resemble somatic embryos" from protoplast derived calluses.

As we have seen the extensive studies of Negrutiu et al. (1975; 1978a; 1978b; 1978c), Gresshoff and Doy (1972) and Corcos et al. (1973) have been instrumental in determining the type of culture systems used by those attempting to regenerate from tissues of A. thaliana. Although still used (for example Zhang and Somerville (1987) and An et al. (1985)), this type of culture regime is not ideal, especially if the recovery of genetically engineered tissues is the desired end point. In such cases regenerants are required rapidly and with as little risk of genetic modification by the in vitro conditions as possible. The Negrutiu-type system has a large explanting to regeneration time

interval (up to three months) involving a very long callus phase with its inherent problems of genetic instability (D'Amato, 1977) and loss of regenerative potential (Negrutiu et al., 1978b; Halperin 1986). Recently, therefore, attempts have been directed towards designing regeneration systems that will reduce the duration of the callus phase and the regeneration time whilst improving upon the morphogenic frequencies obtained by previous workers.

During the last few years some success has been achieved in this direction. Acedo (1986) developed a two-stage culture for seedling explants in which 2,4-D alone was used in the callus induction medium and NAA and BAP in the shoot regeneration medium. Although the NAA concentration is remarkably high, (four times higher than that of the BAP) regeneration frequencies of 80% for Landsberg and 60% for Columbia were achieved after three weeks in the first and an undisclosed time in the second stage. Interestingly, MS was found to be superior to B5 (the basal medium favoured by Negrutiu) for callus formation and regeneration in this study.

It is the work of Feldman and Marks (1986), however, which has achieved most in modifying the approach to Arabidopsis culture systems. They designed their experiments around what they called "the short preculture concept". The intention of this was to reduce the time in culture, especially the duration of the callus phase, and so minimise the problems associated with long-term cultures described above. They demonstrated that for their system a relatively short period (only seven days) in the

callus induction medium was optimum, and that after this time transfer to a shoot induction medium supplemented with 8.5 x 10^{-7}M IAA and 2.5 x 10^{-5}M 2iP induced up to 100% of the calluses to undergo caulogenesis. Further, each responding callus was capable of producing, on average, 3.9 shoots. Increasing the first stage beyond seven days reduced the caulogenic potential of the callus tissue.

Under the above conditions these authors found that some media used in previous studies were not very effective: DBM (Gresshoff and Doy, 1972) favoured callus development and PG3 (Negrutiu et al., 1975) root production respectively, at the expense of shoot formation. They also found the genotype Columbia to be relatively recalcitrant, being capable of only 60% shoot regeneration, a caulogenic potential significantly lower than that of the other two genotypes investigated.

Three recent studies (Patton and Menke, 1988; Gleddie, 1989; Damm and Willmitzer, 1988) have further expanded our knowledge of caulogenesis in Arabidopsis. In the first Patton and Menke (1988) regenerated shoots within 10 days when placing pre-desiccation stage zygotic embryo parts from Columbia on B5 medium supplemented with 10:1 BAP:NAA. Regeneration frequencies of up to 60% were obtained from the cotyledons but much lower values from the hypocotyls and mature leaves. In the second of these papers Gleddie (1989), also using Columbia, provides the first detailed report of plantlet regeneration from suspension cultures. Cell colonies from the suspension were plated onto differentiation media containing a range of cytokinin types and concentrations, and

although no time scale is given for the regeneration, it was found that thidiazuron was the best cytokinin source followed by zeatin and BAP. Gleddie was also the first since Negrutiu to look at temperature effects and was able to show that optimum regeneration took place at 20°C and below, not at the more usual incubation temperature of 25°C.

Lastly, Damm and Willmitzer (1988) described the most successful system yet for the recovery of fertile plants from protoplasts. In their regime shoots were regenerated from microcalluses using a number of induction media; the most successful containing BAP and NAA or iPA and IAA. This system was devised especially for Columbia and some very high frequencies were reported (87%); the highest to date for this genotype.

Aims of the Current Work

As we have seen, except for a few very recent reports, the protocols for organogenesis from *A. thaliana* are based largely on the studies of Negrutiu *et al.*, in which regeneration is restricted to recovery from multi-step, long-term callus cultures while the production of somatic embryos is restricted to one low frequency system (Huang and Yeoman, 1984). With *Arabidopsis* becoming increasingly important as a model for genetic and molecular genetic studies it was considered to be imperative to investigate its *in vitro* morphogenetic characteristics further.

The aims of this work were therefore to study the factors controlling morphogenesis in this species to provide information that could contribute to the development of regeneration systems

with a more rapid, higher frequency response than have been available to date. It was decided to concentrate on both organogenesis and embryogenesis as it was considered that the latter especially was poorly understood. It was hoped that such an approach would provide regeneration systems more suited to the needs of the molecular geneticists and genetic engineers and secondly to provide insights regarding the developmental controls behind plant morphogenesis. The special attributes of A. thaliana described earlier provide a unique opportunity to study aspects of plant development at the gene level, but before such correlations can be made as much as possible must be known concerning its morphogenic characteristics.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Since the pioneering discoveries of Skoog and Miller (1957), Steward *et al*. (1958) and Reinert (1958) numerous potential applications of procedures involving *in vitro* plant regeneration have become apparent. These include:

1. the rapid clonal multiplication of crop plants that are either difficult or slow to reproduce by conventional methods, or of desired genotypes which are lost by obligatory outcrossing.

2. the production, and subsequent multiplication, of pathogen- free stock plants (Henshaw, 1979; Smith and Drew, 1990).

3. the generation of haploid and genetically altered plants for inclusion in breeding programs (D'Amato, 1977; Evans *et al*., 1981; Bajaj, 1983; Cocking, 1987; Smith and Drew, 1990).

4. long term germplasm storage (Hussey, 1978; Sakoi, 1987). Indeed, Murashige correctly predicted in 1974 that "Routine employment of tissue cultures as an alternative method, in some cases the only method, of commercial propagation of diverse herbaceous plants will become a reality very soon".

The phenomenon of *in vitro* regeneration occurs either by adventitious or non-adventitious formation of new organs or somatic embryos from the cultured plant material. Micropropagation, the most important current application of plant tissue culture, generally exploits the former, whereby shoots are produced by suppressing apical dominance and encouraging the development of the

axillary buds. This is an example of non-adventitious regeneration since the plants are produced from the non-adventitious axillary buds. In adventitious systems organs or embryos are formed in abnormal places (Hussey, 1978), for example; shoots from roots, roots from callus tissue or embryos from anthers.

Although many horticultural crops are now routinely multiplied by micropropagation (Murashige, 1974; Hussey, 1978) the adventitious regeneration systems are potentially of greater value. This is because they are not restricted to using shoot meristems, and they can, theoretically, regenerate almost unlimited plantlets from any explant source. This is necessary if *in vitro* morphogenesis is to contribute further to agricultural development. The process of adventitious plant regeneration from callus tissue also facilitates the recovery of genetically altered plants from the parent tissue. Whether altered via somaclonal variation, induced spontaneously by the culture conditions or by the introduction of desired traits by genetic engineering or from haploids via anther cultures, these regenerants are valuable as they can be screened for their beneficial qualities and subsequently introduced to crop improvement programs.

Finally, the formation of adventitous organs and embryos raises many fundamental questions regarding the regulation of plant development, while simultaneously providing a valuable experimental tool for the investigation of this important aspect of plant science (Halperin, 1973).

Adventitious regeneration can take place by two distinct processes: organogenesis or embryogenesis. During organogenesis a root or shoot meristem is formed which, if provided with the correct conditions, is capable of developing into the mature organ; this new organ is continuous with, and has vascular connections with, the parent tissue at it's basal end. In somatic embryogenesis, however, a distinct bipolar plumular/radicular structure is formed which has no vascular connection with the parental tissue (Haccius, 1978; Ammirato, 1985). Both of these processes can take place with or without the intermediate formation of callus tissue, by what Hicks (1980) defines as direct or indirect regeneration respectively.

Although adventitious regeneration has great potential for crop improvement, especially embryogenesis with the small size and complete form of the resulting propagule facilitating possible mechanisation of the procedure (Lutz et al. 1985; Styer, 1985), a number of factors have hindered the realisation of this promise. Firstly, many important plant groups such as the cereals, legumes and tropical and forest crops will not regenerate with the ease of the test or model species (Vasil and Vasil, 1980; Evans et al., 1981; Litz, 1985; Lutz et al., 1985) and secondly mature tissues from genetically proven, desirable genotypes are often difficult to manipulate in culture (Sharp et al., 1980). The ability to perform in vitro is genetically controlled and in many cases regeneration from valuable tissue may prove impossible. Furthermore, the genetic uniformity of the resulting plants is often questionable

(D'Amato, 1978), and while this may be of use for generating breeding material it is unacceptable for clonal multiplication purposes.

Although considerable progress has been made recently with some crop plants, notably the cereals (Vasil and Vasil,1980; Vasil, 1985) and the legumes (Meijer and Brown, 1987; Grica et al., 1987), the regeneration systems reported are often low in the quality and/or the quantity of the resulting propagules or they require lengthy and complicated culture systems that are often only transiently productive, or produce plants of unreliable genetic consitution. With new and improving techniques for manipulating single cells in culture - such as protoplast fusion (Exking, 1987; Evans, 1989), microinjection and genetic transformation (Kumer and Cocking, 1986; Thomas and Wernicke, 1978; Cocking, 1987) - the ability to recover plants from specified material at will, becomes of paramount importance and gives plant tissue culture a pivotal role in future crop improvement programmes. At present we do not have this ability and it is therefore essential that research into all aspects of morphogenesis continues apace, especially into its underlying developmental processes. Only by understanding the fundamental aspects of organised development can regeneration be achieved on demand in diverse plant materials (Thorpe, 1982; Tran Than Van, 1981a).

The aims of this thesis are directed towards such an end and can best be illustrated by first briefly examining the body of literature available on morphogenesis. This will be split into two sections; firstly a consideration of the key variables of practical

tissue culture, which are the researcher's major tools, followed by a review of what is currently understood about the developmental processes behind *in vitro* regeneration.

The Variables of Plant Tissue Culture

Most research into *in vitro* morphogenesis has revolved around manipulating the numerous variables available to the tissue culturalist (Street, 1979; Thorpe, 1980). The number and variety of these is considerable but they can be separated into three main types: the explant source, the composition of the culture medium and the physical conditions of the culture system (Tran Than Van, 1978; Brown and Thorpe, 1987).

1. The Explant Source.

i. Explant type

Almost any living part of the plant can be brought into culture and induced to form callus (Narayanaswamy, 1977; Ammirato, 1983a). If one wishes to achieve morphogenesis, however, the choice of explant is critical, and is considered by many to be the most important variable determining the success of a culture system (Tran Than Van, 1978; Tisserat *et al.*, 1979; Street, 1979; Sharp *et al.*, 1980; Vasil, 1985). Generally, juvenile tissues and those containing meristematic or actively dividing cells are the best explant sources. These include shoot apices, embryos, immature infloresences, the nucellus, cambium tissues and seedling parts, especially the hypocotyl (Tisserat *et al.*, 1979; Gamborg and Shyluk, 1981; Murashige and Huang, 1985; Williams and Maheswaran,

1986). Evans et al. (1981) list the frequency of use of each of these explant types for direct and indirect somatic embryogenesis and states that hypocotyls are used in 44% and 56% of successful regeneration systems respectively.

The regenerative potential of any type of explant varies from one species to the next. In the model systems of tobacco and carrot regenerants can be obtained from almost any plant part (Ammirato, 1983a). For most species, however, effective explants are limited (Flick et al. 1983); in grasses only immature embryos, young infloresences and leaf sheaths are morphogenic (Vasil, 1982; 1985) and for tropical fruit trees somatic embryos are obtainable from nucellus integuments and zygotic embryos only (Litz, 1985). Furthermore, the size, shape and exact location of the explant, even within one organ type, can have considerable influence on that culture's regenerative capacity (Murashige, 1974; Narayanaswamy, 1977; Tran Than Van, 1978; Yeoman, 1970; Bapat and Rao, 1984; Ammirato, 1985).

Lastly, the ontogenetic stage of the mother plant at the time of explanting is critical. Factors such as dormancy, juvenility and the onset of flowering all affect the physiology of the mother plant tissue and determine the explant's reaction to the culture system and its regenerative potential. (Tisserat et al., 1979; Tran Than Van, 1978; Sharp et al., 1980; Evans et al., 1981; Vasil, 1985).

ii. Genotype

Receiving less attention than the explant type in past reviews, but of equal importance, is the role of the genotype in obtaining a successful culture (Evans et al., 1981; Ammirato, 1983; Brown and Thorpe, 1986; Halperin, 1986). Similar explants in identical culture conditions can have significantly different regeneration potentials depending on the genetic background of the plant material. Studies on sorghum (Brettel et al., 1980), sweet potato (Jarret, 1984), cotton (Shoemaker et al., 1986), alfalfa (Meijer and Brown, 1987; Chen et al., 1987), maize (Lu and Vasil, 1982), wheat (Carman et al., 1987) the brassicas (Dietert et al., 1982; Narasimhulu and Chopra, 1988), soybean (Christianson, 1985) and Arabidopsis (Negrutiu et al., 1975), among others, illustrate this fact.

Such variation operates at the species level and below; even down to specific genotypes. Chen et al., (1987) screened 50 genotypes from three different alfalfa cultivars and found differences in embryogenic potential between and within the cultivars, and Negrutiu et al., (1975) reports similar results for organogenesis between genotypes of Arabidopsis thaliana. Thus within many species there are responsive and recalcitrant genotypes; often one, or a few genotypes, of an otherwise amenable species will refuse to regenerate regardless of variable manipulations, or for some reason only one out of a largely recalcitrant type will be morphogenic. There is no way of predicting this prior to culturing and thus each genotype must be tested empirically.

2. The Culture System

Once an explant's type and genetic background has been chosen it is placed into the culture system. This consists of numerous physical and chemical variables all of which can be manipulated by the worker and all of which may have an effect on the type and degree of morphogenesis.

i. Physical variables

The most important of the physical variables are temperature, light quantity and quality, gaseous environment and the pH. Although most workers keep these fixed and concentrate on manipulating the culture's chemical environment, all, especially light and temperature, have been shown to influence morphogenesis. Hughes (1981) reviews this area thoroughly and stresses the importance of photoperiod and spectral quality, while others have found the total daily dose of radiation to be the most important factor (Murashige and Huang, 1985). A common temperature employed for incubation of the culture is 25°C, but rarely is this optimised for each tissue type. Generally the smaller the explant the greater is the effect of the physical variables (Tran Than Van, 1978) but ideally, as for the selection of the explant, these should be ascertained empirically for each tissue under investigation.

ii. Chemical variables

The chemical composition of the culture medium can be divided into that of the basal medium and its growth regulator additions.

The basal medium

The basal medium is designed to supply all the inorganic nutrients required for healthy *in vitro* growth. The various basal media used in plant tissue culture differ only in the exact concentration of these inorganics and the ion-complexes in which they are supplied (Narayanaswamy, 1977; Flick, *et al.*, 1983; George and Sherrington, 1984). Considerable efforts have been made in developing effective basal medium and Narayanaswamy (1977) lists the ten most commonly used today. Of these Murashige and Skoog (1962) is the most popular being employed in 70% of all recent studies (Evans *et al.*, 1981).

All modern basal media contain a reduced nitrogen source and while it is recognised that *in vitro* morphogenesis is primarily controlled by auxin and cytokinin, the importance of reduced nitrogen in embryogenesis, and to a lesser extent organogenesis cannot be ignored. Halperin and Wetherell (1965) were the first to cite the critical role of nitrogen by reporting that the addition of ammonium ions to the medium significantly increased the number of embryos produced by carrot cultures. Reinert *et al.*, (1967) found that high levels of nitrate ions could also promote embryogenesis, but since that time the reports of Halperin have been confirmed in carrot, and other species, while those of Reinert have not. It is now accepted that a source of reduced nitrogen

must be included in the medium if high frequency somatic embryogensis is to occur (Kohlenbach, 1978; Stuart et al., 1985, Meijer and Brown, 1987).

There are a number of different forms in which the nitrogen may be added to the medium. It can be supplied as complex additions such as the casein hydrolysate, as specific amino acids, singly or in mixtures, or as the ammonium ion itself (Ammirato, 1983). Such studies have shown that while the inclusion of arginine, aspartic acid, glutamine and especially the aromatic amino acid tyrosine are beneficial for organogenesis (Murashige, 1974; Thorpe, 1982) it would appear that most basal media contain nitrogen in sufficient amounts and in the correct form(s) to facilitate organ formation. Successful embryogenesis, however, is considerably aided by nitrogenous additions. In carrot the most effective source is that of glutamine, and to a lesser extent alanine (Whetherell and Dougall, 1976; Kamada and Harada, 1979; 1983; 1984), although in alfalfa proline is also highly promotory, while Stuart et al., (1985) have shown that to be effective these must be added at concentrations above 30mM.

In somatic embryogenesis the requirement for exogenous nitrogen does not exist in the first stage; embryogenic cells are produced in the presence of auxin only (Sharp et al., 1981; Kamada and Harada, 1983), but it is in the second stage, where development proceeds from the globular to the cotyledonary stages, that high levels of nitrogen are most beneficial. Added at this time it significantly improves the quality and quantity of the resulting embryos.

The growth regulators

The addition of plant growth regulators, notably auxin and cytokinin, along with the explant source are the most important of the culture variables affecting *in vitro* morphogenesis. The type and concentration of these two categories of growth regulators and the explant source can determine whether regeneration takes place via organogenesis or embryogenesis.

Although direct morphogenesis can occur in culture (Evans *et al.*, 1981), in most cases a medium with high auxin levels alone, or often in combination with a low level of cytokinin, is used to induce cell division and callus formation by the explant. The regeneration event then takes place from this callus tissue.

a. organogenesis

Skoog and Miller (1957) were the first to demonstrate the ability of auxin and cytokinin to stimulate root and shoot formation respectively. Research during the following two decades then allowed Kolhenbach (1977) to make five statements that neatly summarise our knowledge concerning the action of growth regulators on organogenesis. These are:

1. Removal of auxin. After a preculture with auxin its removal often leads to root formation. Examples: carrot (Reinert, 1958), *Atropa belladonna* (Thomas and Street, 1970).

2. Ratio of auxin to cytokinin. A high ratio of auxin to cytokinin favours root formation whereas an opposite ratio favours shoot buds. Examples: Petunia (Durand et al., 1973), Macalega cordata (Lang and Kolhenback, 1975), tobacco (Skoog, 1971).

3. Absolute concentration of phytohormones. Examples: Lycopersicum leaf callus (Padmanabhan et al., 1974) where $2mgl^{-1}$ IAA + $2mg^{-1}$ kinetin causes root formation and $4mg^{-1}$ IAA + $4mg^{-1}$ kinetin gives shoot formation.

4. Nature (type) of the auxin and cytokinin. Examples Asparagus callus (Bui Dang Ha, 1974) where shoots arise in the presence of BAP in combination with IAA or NAA but not in media containing zeatin or 2,4-D.

5. Phytohormones other than auxin and cytokinin. Examples: tissue derived from sweet potato and potato (Yamaguchi and Nakajima, 1974) where shoot buds may be produced by replacing kinetin with ABA.

These statements may contradict each other to some extent, but this is useful in illustrating the diversity of response shown by different tissues. For some, the ratio of the two growth regulators is critical, for others it is their absolute concentration. In further cases this is complicated by a specific preference for one kind of auxin or cytokinin and/or the influence of a third factor. As a result each tissue type, that is every explant source, must be investigated empirically to determine its optimum regeneration regime.

b. Embryogenesis

Shortly after the discoveries of Skoog and Miller (1957) Steward et al. (1958) and Reinert (1958) independently reported the production of somatic embryos from cultured carrot tissue. This original work plus that of subsequent researchers, has, as for organogenesis, allowed some general conclusions to be made as the role of the growth regulators in this process.

Auxin is the prime controlling factor in embryogenesis, and successful cultures are obtained by treating explants with various types and concentrations of this growth regulator. However, although auxin will induce the formation of an embryogenic callus, continued exposure to high levels promotes cell division at the expense of organised development. As a result, transfer to a medium with reduced auxin is a prerequiste for embryo formation (Reinert et al., 1967; Wetherell, 1977; Vasil and Vasil, 1980; Evans et al., 1981; Ammirato, 1983; 1985). Reduction of the auxin can be achieved by its total removal, lowered concentration or replacement in the second stage with a weaker type.

As with organogenesis the type of auxin used can also be important. Some tissue, for example those of carrot, will produce callus and regenerate regardless of the auxin source, but for most a specific type and concentration is necessary (Yeoman and Forsche, 1980; Ammirato, 1985). 2,4-D has the strongest auxin action and used at concentrations between 10^{-7}M and 10^{-5}M is effective at promoting callus formation from most tissues (Hussey, 1978; Yeoman and Forsche, 1980). Evans et al. (1981) report that 2,4-D is used in the first stage of 57% of all successful embryogenic cultures

and in many cases, most notably that of the grasses, only 2,4-D has the ability to induce embryogenically competent tissue (Vasil, 1985; Vasil and Vasil 1980).

While auxin is essential for embryogenesis the role of cytokinin is less clear. In some cases it is needed for the successful formation of an embryogenic callus (Wetherell, 1977; Evans et al., 1981) but in many its inclusion may be ineffective or even inhibitory (Vasil 1985; Wetherell, 1977; Kohlenbach, 1978; Ammirato, 1983a). In the second stage Evans et al., (1981) state that cytokinins were used in about one third of reported embryogenic cultures, but very rarely (6%) were they used alone, as would be the case for caulogenesis (Evans et al., 1981; Ammirato, 1983a; 1985). Thus the cytokinins have a minor role to play in embryogenesis compared to that in organogensis where they are usually essential, in some form, for shoot formation.

Other growth regulators have been added to the basal medium in attempts to improve regeneration frequencies and/or the quality of the resulting plantlets. The most important of these are GA and ABA.

GA is not commonly employed in either the first or second stage but it is reported to enhance shoot formation in Chrysanthemum (Bush et al., 1976) and Arabidopsis (Negruitu et al., 1978a). These cases would seem to be the exception, however, as in most reports it is considered to be inhibitory to morphogenesis, and especially caulogenesis (Thorpe and Meier, 1973; Street, 1979; Thorpe, 1982; Ammirato, 1983).

Generally, if GA is added its use is restricted to stages after the morphogenic event where it is used to enhance the growth and development of the newly formed plantlet (Murashige, 1974; Ammirato, 1983).

ABA, when used at low concentration, may aid callus formation and caulogenesis (Street, 1979). Certainly it can reverse the inhibitory effects of GA (Thorpe and Meier, 1973; Murashige and Huang, 1985) and promote caulogensis in potato (Shepherd, 1980) and sweet potato (Henderson, et al., 1984). The major role for ABA is, however, that of a "normalising" factor in the development of somatic embryos. As demonstrated by the work of Ammirato on caraway (1977; 1983b), if supplied after the onset of embryogenesis (ie. in the second stage), and at concentrations below those inhibitory to growth, ABA will reduce precocious germination and aberrant embryo formation thereby significantly improving the quality of the resulting propagules.

Plant Tissue Culture and its Use in the Study of Plant Development

The promise of economic returns, coupled with its apparent practical simplicity, has led most workers to concentrate on the empirical approach to *in vitro* regeneration. This is what Street (1979) calls "ringing the changes" and is based on manipulating the culture parameters described above, especially that of the growth regulators, until success is achieved. This has resulted in the accumulation of information regarding the practical aspects of morphogenesis rather than the underlying processes behind it (Street, 1979). Further, the successes reported are, most often, from limited tissue types at levels or qualities below those required for realistic commercial exploitation. Thus at the moment our understanding of the mechanisms controlling morphogenesis are poor, as are many of the systems devised to exploit it. Only by studying the fundamental developmental aspects of this processs will our understanding of *de novo* regeneration increase, allowing practical tissue culture to advance to the point where regeneration "at will" becomes a reality (Thorpe, 1982; Street, 1979). In addition, such information can only facilitate greater understanding of *in vivo* plant development at the cell, organ and whole plant levels (Henshaw, *et al.*, 1982; Christianson, 1987).

The purpose of this section is to examine the current concepts behind *in vitro* regeneration. Much of the present understanding of plant development is theoretical in nature but does relate to the practical aspects of tissue culture as already outlined.

Murashige (1974) lists the three stages required for production and recovery of regenerants. These are, the establishment of an axenic culture capable of sustained cell proliferation and growth, the regeneration or multiplication of the desired organ or plant structure and finally the rooting and hardening of the recovered propagules prior to transplanting to the soil. Street (1979) states the function of the first as "one that leads to the appearance of actively proliferating callus" and the second as "....the development....of organised growth leading to organogenesis or embryogenesis,....a satisfactory morphogenic medium is one in which morphogenic expression is rapid and prolific". Thus one starts with an explant in which the cells are induced to divide, and it is from these callus cells that the new organs are formed. The anatomy of callus growth and the patterns by which the new structures develop will be examined in some detail in Chapter 2; here we will discuss these events at the levels of the tissue, the cell and below.

The theory of totipotency is an old one, dating back to the Cell Theory of Schlinden and Schwann and it is based on the idea that every living cell in the plant has the ability, if provided with the correct conditions, of developing from a single cell to the whole plant (Steward, 1970; Henshaw *et al.*, 1982).

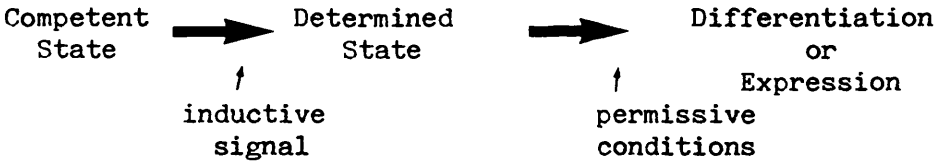
Presently, there are two schools of thought concerning the role of the culture regime in facilitating the expression of this totipotency. Some believe that the cells of the explant, or some of them, have the inherent ability to develop into new structures, and the processes of excision and culture act only to allow this potential to be expressed. They consider that it is not possible to induce such properties in culture but that the in vitro conditions, growth regulator concentrations etc., are conducive to the cloning of these cells in the first stage culture, and expression of their potential in the second (Tisserat et al., 1979, Street, 1979).

Others attribute greater importance to the culture system, stating that by its action, cells of the explant not inherently capable of organ formation can be induced to be so by the action of the culture system (Sharp et al., 1980; Sharp et al., 1982). These are what Sharp et al., (1980) (with respect to embryogenesis) calls predetermined embryogenic cells (PEDCs) and induced embryogenic cells (IEDCs) respectively. In the former the cells have been determined for embryo production prior to explanting, while in the latter they were not determined for this fate until induced to be so by the action of excision and culturing.

We will return to this later, but at this point it is necessary to define the terms determination and induction and the associated theorectical states of plant development. Figure 1 shows the relationship between the competent and the determined

states as defined by those studying animal development (Waddington, 1966). These definitions can be applied at various levels of organisation: cell, tissue of organ.

Figure 1 The Relationship between the States of Competence, Determination and Differentiation.



Competence is the capacity or ability to express an inherent potential in response to appropriate inductive signals and permissive conditions; in the absence of the permissive conditions, the cell, tissue or organ may remain in the stable determined state which can be transmitted clonally but not meiotically (Henshaw et al., 1982; Sachs, 1978). Determination is the procress by which the developmental potential of a cell, tissue, or organ becomes limited to a specific fate which will be expressed, given permissive conditions. Our present level of understanding is such that both the competent and determined states are cryptic and they can only be identified in terms of structural, physiological or biochemical response to specific conditions.

The concepts of determination, competence, induction and differentiation were introduced by those studying animal development. There are, however, a number of experimental systems

which demonstrate that these states can also be applied to many aspects of plant development (Henshaw et al., 1982; Sussex, 1982; Meins and Binns, 1979; Christianson, 1985). Space precludes examination of these in detail, but they include, stem and branch determination in Norfolk Pine (see Meins and Binns, 1979), the juvenile and adult phases of Hedera helix (Banks, 1979; Meins, 1986), Osmundia fern leaf and stem formation (Meins, 1986) and floral determination of apical meristems (Sussex, 1982).

In one of the few studies to tackle these rather theoretical concepts Christianson and Warnick (1983; 1984; 1985) demonstrated that in Convolvulus the states of competence, determination and differentiation can be separated and identified experimentally. They achieved this by culturing leaf tissue for differing times on callus ($3mgl^{-1}$IAA/$0.3mgl^{-1}$K), root ($12mgl^{-1}$IAA) and shoot ($0.05mgl^{-1}$IAA/$7mgl^{-1}$2ip) induction media followed by transfer to a hormone free basal medium. With no growth regulators present in this final medium, only tissue determined for a specific morphogenic state at the time of subculture to it, will be able to express that fate and undergo differentiation. By varying the sequence and duration of exposure to these media, especially the callus induction medium, these workers claim to have identified the timing of the states of organogenic competence and determination in this tissue. Further, through the use of a number of inhibitors; TBA, sorbitol, acetyl salicyclic acid and ammonium (Christianson and Warnick, 1984), which arrested the organogenic processes at

different stages, they claimed to have described at least five discreet directive inductions in *de novo* regeneration in *Convolvulus*.

All of these systems illustrate determination for organ formation by pre-existing organs and tissues, but as yet there is little evidence to indicate morphogenetic determination at the level of the individual cells (Henshaw *et al.*, 1982). It has been claimed, however, that determination at the cell level is demonstrated *in vitro* by the phenomenon of habituation, whereby cells which require added growth regulators for active growth can lose this requirement and thereafter produce sufficient hormones for continuous growth without supplements (Meins, 1986). It has been shown that this adaptation from auxotrophy to autotrophy is not due to a mutation but is is gradual, directed, stable but reversible and transmitted clonally but not meiotically (Binns and Meins, 1973; Lutz, 1971). Thus it is an epigenetic change - a change in gene expression not gene complement - and it therefore follows that determination and differentiation are also epigenetic in nature (Sussex, 1982; Meins, 1986).

How then does this theoretical approach relate to the practical aspects of *in vitro* morphogenesis? Tran Than Van (1981) uses the term "inherent cellular state" to describe the intrinsic properties of a given cell or tissue. This covers a multitude of unknowns (Thomas and Wernicke, 1978), such as the epigenetic state, endogenous growth regulator concentrations and nutritional status, and it effectively labels the plant material as a "black box" where the worker can simply manipulate the inputs and record the outputs

without understanding what takes place inside. The term "inherent cellular state" is therefore pragmatic since it encompasses not only the states of competence and determination - without assuming that they exist in a particular cell or tissue - but also more transient states.

Any explant has an inherent cellular state dependent on that tissue's past history; this includes the genotype, age and physiological state of the mother plant and the explant type. It has been demonstrated in a number of cases that cultured tissues do not consist wholly of cells with a ground-state or dedifferentiated phenotype, and that even after considerable time in culture they can "remember" their past so affecting their morphogenetic plasticity (Meins, 1986). For example:

a. in tobacco only explants from plants already induced to flower can regenerate meristems determined for flower formation (Tran Thanh Van, 1981).

b. in Hedera helix differences in the regenerative capacities of tissues from the juvenile and adult phases persists indefinitely in long term culture. Juvenile tissue grows faster (Stoutmeyer and Britt, 1965) and forms roots and shoots while adult tissue initiates somatic embryos (Banks, 1979).

c. pith, cortical and leaf explants of tobacco show different levels of cytokinin habituation and patterns of organogenesiss (Meins and Lutz, 1979; Meins and Binns, 1979).

d. in some cultures, organ-specific proteins have been detected in cells after numerous subcultures (Raff et al., 1979; Arnison and Ball, 1975).

In these examples some inherent cellular factor, be it epigenetic or otherwise, has been passed through explanting and considerable cell multiplication, to influence the competence of the resulting culture. If these factors are inhibitory to morphogenesis than such tissues will have a low or zero regeneration potential (Halperin, 1986).

Interestingly, epigenetic traits are not conserved through meiosis (Meins, 1986; Sussex, 1982; Street, 1979), nor by definition is the state of determination. The end products of meiosis, the gametes and the zygote are certainly totipotent, as are, to differing degrees, the associated tissues. Indeed the proven, large regenerative potentials of such explants (Evans et al., 1981; Tisserat et al., 1979) indicate that some factor(s) removed at meiosis are at play in influencing the ability to regenerate. Even in the most recalcitrant species juvenile tissues have the greatest morphogenetic potential (Vasil, 1985; Litz, 1985).

Such observations have led some to consider that competence for embryo formation is the basal state of every cell and that any cell released from all constraints "will spontaneously and autonomously express this fundamental developmental pattern" (Wetherell, 1977; Tran Thanh Van, 1981; Henshaw et al., 1982). Meiosis is seen to involve the total removal of all constraints, producing totipotent cells free from epigenetic control.

By this argument, as the ontogenetic age of a tissue moves away from the embryo the cells become determined stop dividing and differentiate physiologically and structurally to

fulfill a specific role. Only the meristematic tissues retain the ability to divide, and in so doing, they produce cells not yet determined for a specific fate (Tran Thanh Van, 1981a). It is no accident therefore that meristematic areas are good explants for establishing morphogenic cultures.

Tran Thanh Van (1981a) considers the progression from competent, meristematic cells to differentiated, non-competent cells to be a gradual one. This is an important concept as it indicates that competent cells could exist in ontogenetically advanced tissues not associated with meristems or the zygote. Evidence for such is provided by somatic embryo formation from single cells of the shoot epidermis in Rannunculus sceleratus (Konar et al., 1972) and Brassica napus (Thomas et al., 1976). In these cases cells which appear to be differentiated have in fact retained the capacity for division and embryo formation. They were still competent for embryogenesis although temporally and spatially removed from the meristematic tissues. It is possible that in some tissues the increase in determination is gradual and/or incomplete and that some of the somatic cells retain their competence for morphogenesis longer than others (Williams and Maheswaran, 1986). If this is a correct interpretation it supports the concept of PEDCS in mature tissues and contributes considerably to our understanding of in vitro and in vivo morphogenesis.

There are a number of tissues, most notably the citrus nucellus and some zygotic embryos which are morphogenic in vivo and in vitro without the need for added growth regulators (Tisserat et al., 1979; Sharp et al., 1980). These are the most obvious PEDCs

and although supplementation with weak auxins and cytokinins increases the incidence of embryogenesis their role is seen as cloning the determined cells rather than inducing newly determined cells. In such tissues the growth regulators need only promote mitosis to elicit morphogenesis (Tisserat et al., 1979).

The concept of IEDCs relies on "...not only the onset of mitotic activity in non-mitotic determined cells but the epigenetic redetermination of these cells." (Sharp et al., 1980). This is suggested to be facilitated by the action of auxin. There is evidence that the auxins, especially 2,4-D can elicit response at the transcriptional and translational levels (Thorpe, 1978; Sengupta and Raghaven, 1979; Theologis, 1989) and possibly may act by unmasking parts of the DNA, so affecting DNA template activity and RNA synthesis (Murashige and Huang, 1985; Sharp et al., 1980). It should be noted, however, that the later view is largely theoretical and although it would fit well with the concept of IEDCs there is little direct experimental evidence for or against it. There is considerable RNA synthesis in response to auxin but that this is due to transcription of newly unmasked DNA is speculative.

Although little is known of the primary action of the growth regulators, more information is available concerning their metabolism and interaction in the cultured tissue. It is clear that the different hormones are not metabolised in the same manner in all tissues, and that this affects their active forms and availability for morphogenetic induction. Jackobsen (1983) treated pea seedlings with auxins and found 2,4-D to be consistently more

effective at inducing callusing than IAA or NAA, and this was correlated with high levels of free auxin in the 2,4-D treated tissue. IAA and NAA were degraded more rapidly and were present as free auxin at much lower concentrations.

Different tissues have also been shown to degrade 2,4-D by different pathways; in the monocotolydons it is metabolised to physiologically inactive glycoside derivitives, but in dicotolydons to physiologically active amino acid conjugates (Feung et al., 1974; 1975). This would account for the former's resistance to 2,4-D applications in the field and their requirement for stronger auxins (or higher concentrations) for morphogenic induction in vitro. Differential growth regulator metabolism has also been shown within the dicots, down to the species and intra-species level in carrot (Montague et al., 1981), apple (Jackobsen, 1981) and Phaseolus (Mok et al., 1982).

One of the major difficulties in designating a specific role for the growth regulators in morphogenesis is that we do not know their mode of action, nor can we be sure of the availability of these compoundds in the tissue (Fosket, 1980). This intra-cell level depends upon the interaction of the amount exogenously applied and endogenously produced. The situation is further complicated when the latter is taken into account. There is, for example, evidence that:

a. added auxins and cytokinins can influence or trigger the endogenous production of each other (Street, 1979).

b. tissues can become habituated for either auxin or cytokinin and this epigenetic change is often gradual (Meins, 1986; Meins and Binns, 1979). Thus when a callus is transferred to an auxin or cytokinin free medium it may maintain supra-optimal endogenous levels of one or the other. Habituated tissues have low or zero regenerative potential (Street, 1979).

c. the exogenous growth regulators are degraded to products with varying morphogenetic activities (Street, 1979).

Taking these factors into consideration it is not difficult to envisage that the physical parameters of temperature, light, gaseous environment etc. will all have an influence on the tissues biochemical systems and so affect the active growth regulator levels and, in turn, regenerative potential.

By inducing cell division, auxins and cytokinins obviously promote DNA replication, but whether or not epigenetic redetermination is achieved by growth regulator mediated action on the DNA complement, or on already existing RNA and protein systems, is not known. If one accepts the IEDC concept, and the equivalent organogenically determined cells, then some redeterimination must take place, but whether this is due to the direct action of auxin on the gene template is unknown. It is possible that the induction of rapid mitosis in cells with weak or incomplete differentiation (Henshaw et al., 1982; Tran Thanh Van, 1981b) is alone capable of producing cells "stripped" of morphogenetic constraint. Clearly this is an area of great interest and potential for future research, as it is basic to an understanding of plant development. Further, if the mechanisms of directive induction, determination

and differentiation can be elucidated then it may be possible to construct systems to reverse this process and regain the juvenile state, thereby rendering recalcitrant tissues morphogenic.

Loss of Morphogenic Potential with Time in Culture

One of the most serious limitations affecting the practical application of *in vitro* technology is the loss of morphogenetic potential with increasing time in culture (Narayanaswamy, 1977, Tran Thanh Van, 1981a; Halperin, 1986). This can shorten the useful life of a culture by reducing the quality and quantity of the regenerants obtainable from it. If the plant material is from a unique explant source then the problem can be critical.

Genetic, epigenetic and physiological factors are all considered to influence the loss of regenerative ability.

Genetic Factors

As a culture's age increases so does the incidence of polyploidy and aneuploidy amongst the cells of the tissue (D'Amato, 1977; Baylis, 1980). Baylis reports that of 55 species studied in long term culture only 11 retained a high frequency of cells with the same chromosome complement as the parent tissue. The extent and type of chromosomal variation depends on the genotype, conditions of culture, especially the 2,4-D concentration (Yeoman and Forsche, 1980), and the degree of organisation of the explant (Meins, 1986).

Loss of morphogenic capacity has been reported in numerous species including tobacco (Murashige and Nakano, 1967), carrot (Reinert, 1959; Halperin and Wetherell, 1965; Smith and Street, 1974), pea (Torrey, 1967) Atropa belladonna (Thomas and Street, 1970), Arabidopsis (Negrutiu, 1976; Feldman and Marks, 1986) and Populus (Coleman and Ernst, 1990) and correlations have been made between this and changing chromosome numbers. Indeed several workers have assessed that these two phenomena are causally connected (Reinert et al., 1967). Certainly the literature contains many examples of the low morphogenic potential of tissues with high ploidy and especially, high aneuploid levels (Halperin, 1986), while Mehra and Mehra (1974) found that only dipliod plants were regenerated from mixiploid callus.

The reason for such poor regeneration from polyploids and aneuploids is not known, but it is has been shown that the cells of these tissues do not maintain the intimate contact and close packing required for meristem and embryo formation. Instead they divide and enlarge so as to be separated from each other or remain only loosely in contact (Halperin, 1986).

Epigenetic Factors

Although correlations exist between increasing chromosomal abnormalities and the loss of regenerative potential, this cannot be the complete explanation. Two factors tell us this: firstly, many diploid tissues will also fail to regenerate after long term culture and, secondly, polyploid and aneuploid regenerants have been obtained from some species (Halperin, 1986).

Sacristan and Melchers (1969) regenerated aneuploid plants from aneuploid callus while similar results have been achieved with rice (Nishi and Mitsouka, 1969), sugarcane (Heinz and Mee, 1971) and Pelargonium (Bennici, 1974).

Such data have generated a view that epigenetic changes, induced by long-term culture, are responsible for the loss of morphogenic potential. We have already discussed habituation, and it is possible that a large proportion of the cells acquire some degree of hormonal autonomy thus affecting the endogenous growth regulator balance and suppressing regeneration. Alternatively, long-term exposure to strong growth factors may well affect the trascription and translation processes so determining the cells for a fate other than morphogenesis (Halperin, 1986).

Physiological Factors

Loss of regenerative ability can, in some cases, be reversed by a change in the constitution of the medium (Yeoman and Forsche, 1980). The clearest reported instance of this is the restoration of embryogenic capacity in long term carrot culture by a high nitrogen addition (Reinert et al., 1967). This illustrates that the loss of regenerative ability need not depend upon alterations of the genome's structure or expression but upon imbalance in the physiology of the tissue. Long-term culture may lead to the depletion of nutritional or other factors below a critical level, and/or a build up of inhibitory substances.

The exact causes, therefore, of a morphogenic decline in long term culture are still not clear, but there is evidence that all three of the factors examined above can have critical effects. It is possible, of course, that more than one of them could be work at any given time in any given tissue.

What can the practical tissue culturalist learn from the theoretical aspects of morphogenesis as discussed above? Firstly, it is apparent that our knowledge of the mechanisms by which the growth regulators and the other variables affect the morphogenetic process is still incomplete. Thus the empirical approach to any new tissue source is still the most efficient way to achieve successful in vitro regeneration. Nevertheless a number of the concepts that have been dealt with above can act as a guide to the empirical approach. Concisely, these are to select explants with as high a compliment of cells competent for a desired fate as possible, as there is no guarantee that the culture conditions can induce competence, and thereafter, to devise a culture system which will select for the multiplication of these cells rather than the non-competent types (Street, 1979). In this manner at the time of subculture, as large a proportion of the cells as possible will be capable of morphogenesis, with the result that a high-frequency highly synchronous response will be encouraged.

At the present time, however, our ability to identify morphogenically competent tissue is limited to an assessment of callus morphology by an experienced eye or by destructive anatomical methods. Even then the morphological and anatomical

characteristics of competent cells are not fully understood and future research must continue to investigate these and to seek biochemical markers and ultimately the mechanisms of gene regulation of the regeneration phenomena. Such knowledge would have profound effects on practical tissue culture as well as our understanding of the control of plant development.

Aims of the Chapter

The experimental work in this chapter describes an empirical study of morphogenesis and the major culture variables that affect it. As stated earlier both organogenesis and embryogenesis were investigated as it was hoped that the regeneration systems reported by previous workers could be improved upon. A range of genotypes were employed, including the two most commonly used laboratory types Columbia and Landsberg. The overall aim was twofold; to design efficient morphogenic systems for possible use by molecular geneticists and for use in studies into the fundamental developmental processes controlling morphogenesis. By utilising a range of genotypes it was hoped to reveal differences in morphogenic capacity and so provide a tool for the investigation of this phenomenon at different levels. While study at the genetic level is outwith the scope of this thesis such data did allow a short investigation of the states of competence and determination for caulogenesis in this system and provided material for the detailed anatomical study of the regeneration process carried out in Chapter 2.

MATERIALS AND METHODS

Materials and Methods

1. Plant Material

Seed was obtained from two sources: a genotype of unknown background through Chambers Seed Specialists and nine named wildtypes from Professor W. J. Feenstra of the University of Groningen, The Netherlands. The latter are listed, with their original country of origin, in Table 1.

Seed were multiplied by surface sowing onto finely sieved Fisons No. 2 compost in 3" pots, watering with a fine rose and enclosing in a polythene bag. The pots were placed under 30$Mm^{-1}s^{-1}$ PAR fluorescent lighting with 16 hrs daylength at 20$\pm$2°C. After five to seven days the bags were removed and the seedlings pricked out into fresh No. 2 compost; thirty to a standard seed tray.

The plants were grown to maturity in the same environmental conditions and the seeds collected just prior to dehiscence of the siliqua. The harvested seed were stored in paper envelopes at room temperature until required.

2. Experimental Plant Material

In order to obtain experimental material seeds were sterilised by immersion in 10% sodium hypoclorite (all details of suppliers are listed in Appendix 1). A drop of Tween 80 was added

Table 1 Genotypes of A. thaliana Studied and their Geographical Origin

Genotype	Abbreviation	Geographical Origin
Columbia	Col.	U.S.A.
Estland	Est.	Germany
Dijon	Di.	France
Chisdra	Chi.	U.S.S.R.
Enkeim	En.	Germany
Landsberg	Land.	U.S.A.
Blanes	Bla.	Spain
Coimbra	Co.	Portugal
Bensheim	Be.	Germany

and the mixture agitated for 30 minutes. The bleach was removed by filtering through sterile No. 1 filter paper and washing five times with sterile water.

Germination took place after transfer to moistened No. 1 sterile filter paper in 9 cm petri dishes sealed with Parafilm. After five to seven days incubation at 25°C + 1°C in 16 hrs day length at 30 $Mm^{-1}s^{-1}$ PAR the cotyledons and hypocotyls were excised with a hypodermic needle and explanted onto the culture medium. Ten specimens were placed in each 9 cm dish or two per well in the case of the 100mm x 100 mm, 5 x 5 square repli-dishes.

To obtain petioles and leaves the seeds were sterilised as above and sown, twenty per petri dish, onto half strength MS medium supplemented with $10gl^{-1}$ sucrose and $7.5gl^{-1}$ agar. After germination the lid was removed and the inverted lower half of another dish sealed with parafilm on top of that containing the seedlings. This permitted unrestricted growth of the plants and excision of the organs as they developed.

3. Culture Conditions

3.1 Media

Murashige and Skoog (1962) basal medium was used in all cultures. This was supplemented with $20gl^{-1}$ Analar sucrose and various concentrations of 2,4-D, BAP, K, IAA and zeatin. In one experiment casein hydrolysate, glutamine and ammonium sulphate were also included, singly or in combination to the basal medium. The pH was adjusted to 5.8 + 0.01 with 1M sodium hydroxide, $7.5gl^{-1}$

agar was added and the medium autoclaved at 1.5 bar/120°C for 15 minutes. IAA was not autoclaved but filter sterilised into the cool sterile medium.

3.2 Culture Environment

After autoclaving the agar medium was poured into 9 cm petri dishes at 25 ml per dish, or into 100 x 100mm square repli-dishes at 4ml per well. All culturing took place at 25°C ± 1°C with a 16h daylength under warm fluorescent tubes at 30 $Mm^{-1}s^{-1}$ PAR.

4. Scoring the Frequency of Response

All observations were carried out under a stereo microscope (Olympus VMX 1 - 4). The response of the culture system was expressed as the percentage of the total replicates reacting in a specified manner. In the case of organogenesis a specimen was scored as positive if it had produced at least one new organ.

5. Statistical Analysis

When replicate numbers allowed the data were statistically analysed. Percentage response were transformed prior to the calculation of Standard Errors and the populations compared using the amended t-test: (Parker, 1979).

RESULTS

RESULTS

1. In vitro Culture of Hypocotyls of Arabidopsis thaliana

Hypocotyls of A. thaliana , obtained from seeds supplied by Chambers Seed Specialists, were excised and cultured on basal medium supplemented with a range of growth regulators. Four major growth regulator interactions were studied: 2,4-D/BAP, 2,4-D/K, 2,4-D/Z and NAA/BAP, and within these the effect of a range of concentrations and interactions were tested by culturing the explants in 5 x 5 square repli-dishes as described in the Materials and Methods. Ten dishes, each containing two hypocotyls per well, were initiated for each of the four growth regulator interactions.

Two experiments were designed: the first to investigate the effect of 28 days continuous culture on the various media, and a second, in which the tissue was transferred after 14 days to a variety of second stage media.

1.1 Reaction of Hypocotyls to a One-Stage Culture System

Figures 1.1 - 1.4 show the degree of callus formation and organogenesis associated with each growth regulator and their interactions at different concentrations. Generally, the hypocotyls behaved in a similar manner in all four of the auxin/cytokinin media tested, although the type and extent of callus formation varied at the respective concentrations.

Four major reactions were recorded.

1.1.1 No Observable Reaction

Failure to respond to the culture medium was observed from three different culture conditions. These were when auxin was absent, when the auxin and cytokinin were used singly or in combination at very high concentration. In the final case growth was prevented at all auxin levels (see Figures 1.1 - 1.4).

1.1.1.1 Hypocotyls cultured without auxin failed to form callus regardless of the cytokinin type or concentration employed. Occasionally they became swollen and green in colour, but usually, and always at high cytokinin concentration, the tissue did not develop; remaining exactly as at the time of explanting.

1.1.1.2 A similar reaction was seen at very high cytokinin concentrations (10^{-4}M), with all auxin concentrations. In this case the tissue turned brown and died 14 to 21 days after explanting.

1.1.1.3 Auxin at very high levels (10^{-4}M) often failed to induce callus; this was especially true when cytokinin was also present at high concentration, and if 2,4-D was the auxin in question. In such cases the hypocotyls either acquired an opaque, white colour, and did not develop further, or turned brown and died during the 28 days culture period.

Figures 1.1 - 1.4 Callus, Root and Shoot Production From Hypocotyl Explants of the Chambers Genotype after 28 Days Continuous Culture on Various Combinations and Concentrations of Auxins and Cytokinins

Hypocotyls were placed on basal medium supplemented with various concentrations of NAA. 2,4-D, Kinetin and BAP as shown. The percentage of the total explant number responding positively is shown, as is the relative size of the callus formed.

Key:

Basal medium	MS supplemented with 2% sucrose
Number of Repicates	40
Callus size	* = minumum to ***** = Maximum
Roots 	
Shoots 	
Callus 	frequency and degree of response

Figure 1.1 Response after 28 Days Culture on 2,4-D and BAP

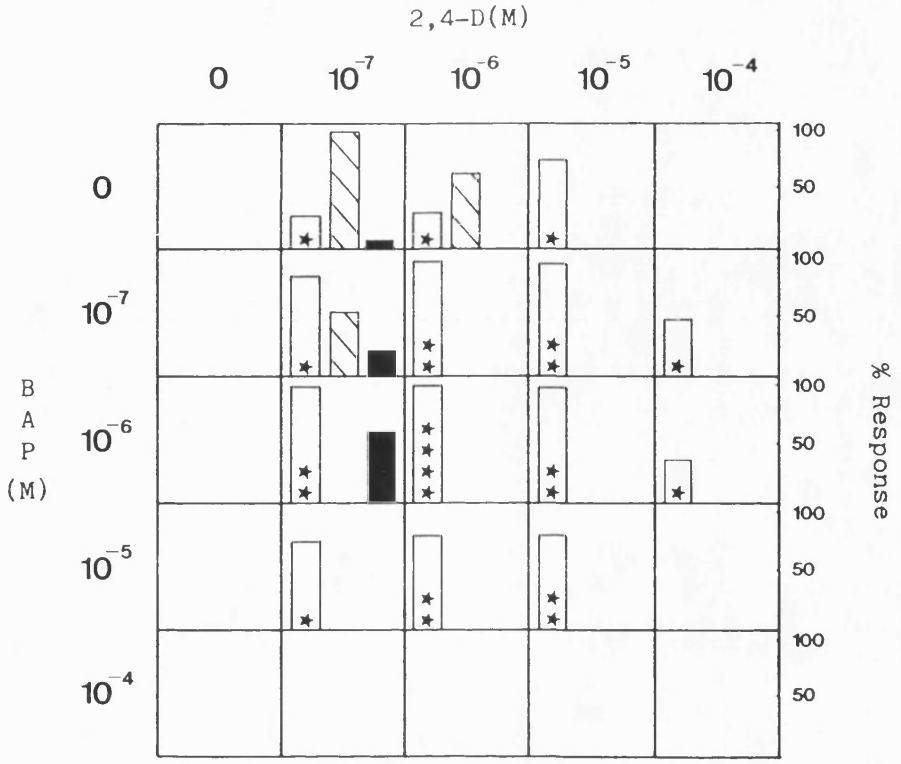


Figure 1.2 Response after 28 Days Culture on 2,4-D and Kinetin

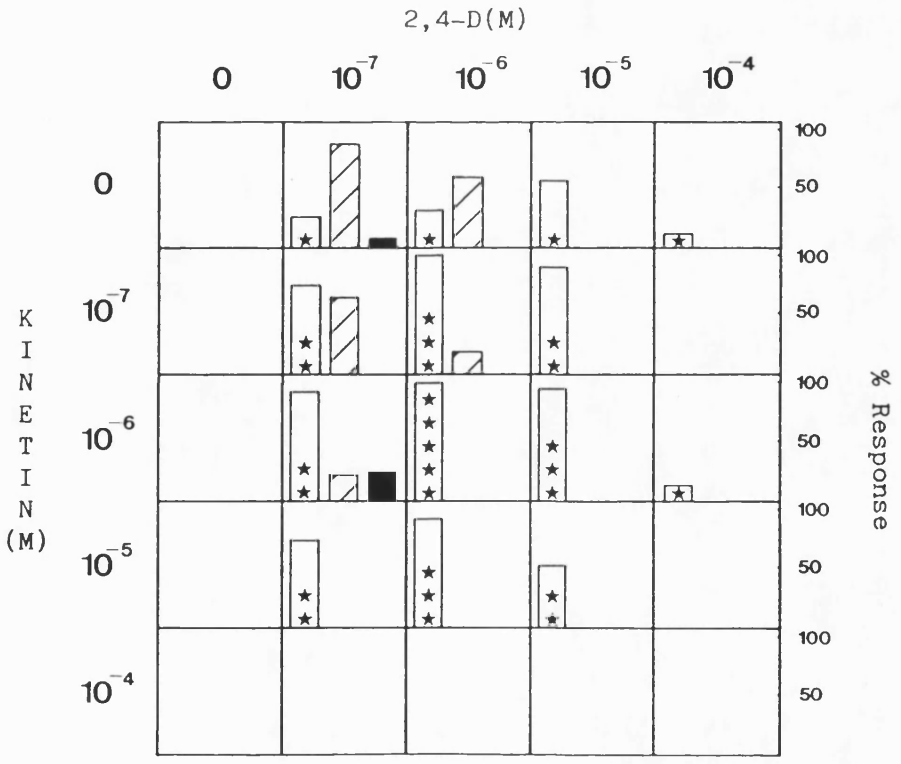


Figure 1.3 Response after 28 Days Culture on NAA and BAP

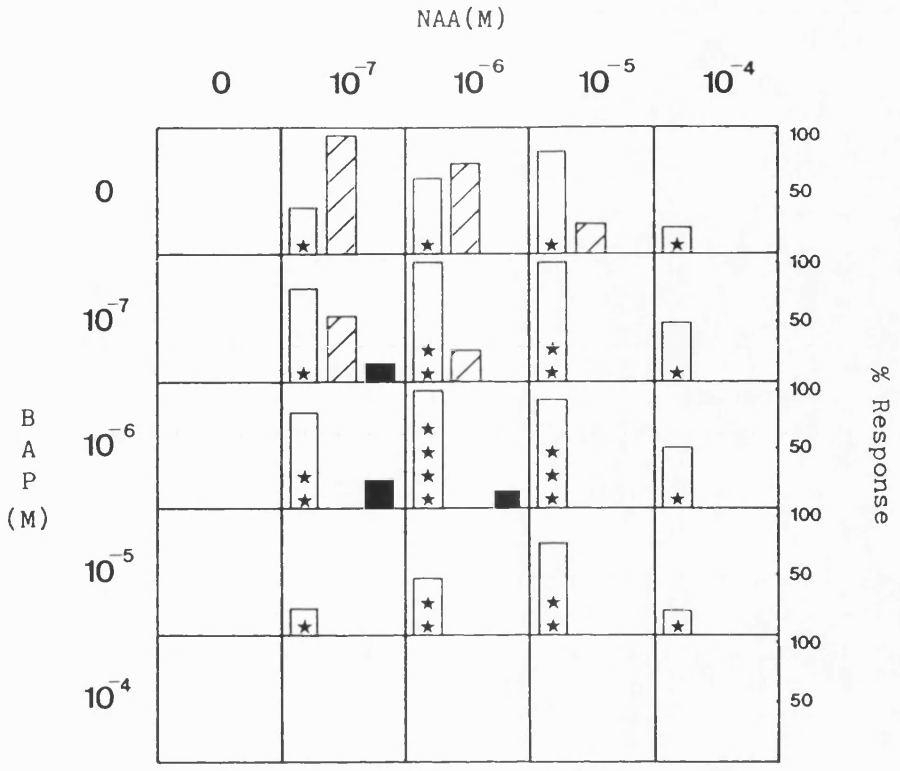
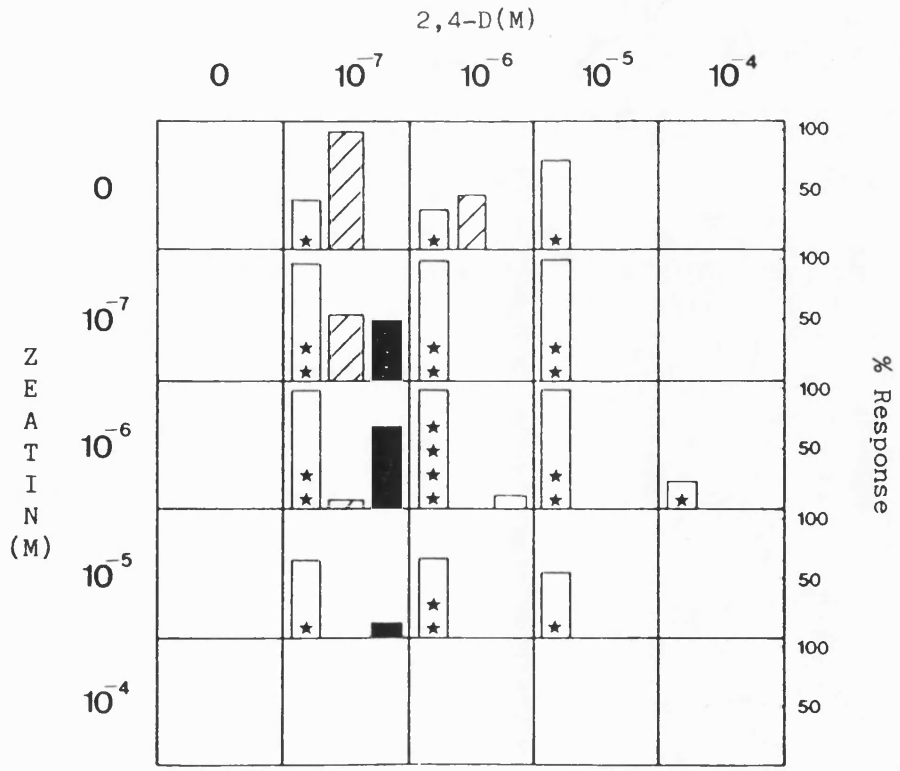


Figure 1.4 Response after 28 Days Culture on 2,4-D and Zeatin



1.1.2 Callus Formation

All four growth regulator interactions were effective at inducing and maintaining callus formation. The overall trends can be seen in Figures 1.1 - 1.4, showing that the percentage response and size of the resulting tissue is maximal at the interaction 10^{-6}M auxin/10^{-6}M cytokinin.

Five major types of callus were seen.

1.1.2.1 Hypocotyls cultured in auxin alone induced only a small amount of callus. This tissue was most often very "hairy" and produced many roots or root-like structures. By the end of the 28 day culture period the majority of the callus present had originated from these root structures, and not from the primary hypocotyl tissue.

1.1.2.2 When auxin was present at low concentration (10^{-7}M) in combination with a cytokinin, small, very green, dry, compact calluses were produced. This was most apparent when 2,4-D was the auxin employed. Callus formed by media containing 10^{-7} 2,4-D/10^{-7}M BAP was "hairy", possessing numerous roots and/or root-like structures. The frequency of these decreased as the cytokinin concentration was increased. The specific interaction of 10^{-7}M 2,4-D/10^{-6}M BAP or zeatin was notable for its deep green, healthy appearance.

1.1.2.3 As the auxin and cytokinin concentration was increased towards 10^{-6}M the degree of callus formation increased. Overall, the combination 10^{-6}M 2,4-D/10^{-6}M K induced the greatest amount of callus, forming a prolific, wet, green and highly friable tissue with no visible organisation. This was apparent, less in the percentage of the explants reacting than in the average size of the resulting tissue. The same concentration of 2,4-D and BAP/zeatin produced a similar callus-type but this was slightly less vigorous in growth. When NAA was used as the auxin source a higher concentration was needed to form such tissue: in this case 10^{-5}M NAA/10^{-6}M BAP, and to a lesser extent 10^{-6}M NAA/10^{-6}M BAP, were effective at producing a friable callus.

1.1.2.4 Auxin at very high concentration (10^{-4}M) formed little or no callus, especially if the cytokinin concentration was also high. NAA was more effective than 2,4-D, however, at this level, see Figure 1.3. This tissue was formed by lateral expansion of the hypocotyl's vascular tissue along its whole length to produce a rough, wet, string-like callus with a white yellow colour. Only about 50% of the replicates developed as such and they grew very slowly. The remainder died from day 14 days onwards.

After 28 days in culture root and shoot formation was seen from tissues cultured on a number of the media. The frequency of these events and the media responsible for them are shown in Figures 1.1 - 1.4.

1.1.3 Root Formation

High-frequency root production was common in all four growth regulator interactions when there was a low to medium auxin/low cytokinin ratio.

Hypocotyls placed on medium supplemented with 10^{-7}M or 10^{-6}M 2,4-D only, produced roots within 14 and 21 days respectively. In the former this took place without the visible, prior formation of any callus tissue, while in the latter, callus production was minimal before the rhizogenic event. 2,4-D at 10^{-5}M failed to form any roots, but NAA at this concentration induced about one quarter of the calluses to form at least one root. The roots formed at these higher auxin levels were thinner, shorter, and less numerous than those produced by tissue cultured on the lower concentrations.

Late in the culture period a proportion of the callus produced on 10^{-7}M auxin/10^{-7}M cytokinin medium formed one or more roots. 2,4-D/K formed the most (70%), but this reaction was common to all four of the growth regulator interactions. When NAA was used as the auxin source roots were also formed by 10^{-6}M NAA/10^{-7}M BAP, but this reaction was at a much lower frequency.

As the concentration of cytokinin included in the medium increased, the potential for rhizogenesis decreased; this was observed in all four of the growth regulator interactions.

Of the two auxins investigated NAA was slightly more effective than 2,4-D at inducing root formation when in combination with BAP, and equally, or slightly more so, when used on its own. All three cytokinins had very similar effects with regard to their abilities to suppress root formation

1.1.4 Shoot Formation

Caulogenesis was a less common event than rhizogenesis, with high frequency shoot formation restricted to only two concentrations of 2,4-D/BAP or 2,4-D/zeatin. 2,4-D in combination with either of these cytokinins induced caulogenesis at higher frequency than media supplemented with NAA or kinetin. There was, however, little observable difference between the abilities of BAP and zeatin to induced high-frequency shoot regeneration (see Figures 1.1 and 1.4).

The most caulogenic growth regulator ratio was 10^{-7}M 2,4-D/10^{-6}M BAP or zeatin which induced 60-65% of the calluses to produce at least one shoot. This ratio was also the most effective when NAA and kinetin were included but the regeneration frequency was considerably lower than that achieved by 2,4-D and BAP/zeatin.

In all cases shoot regeneration was not observed until the fourth week of culture and the number recovered was low.

Shoots were also formed at low frequency on medium supplemented with 10^{-7}M 2,4-D alone, but these arose from the small secondary calluses that grew from the roots produced by the hypocotyl explants earlier in the culture period.

1.2 Organogenesis from a Two-Stage Culture System

In order to study the effect of a two-stage culture system on organogenesis, hypocotyls were cultured on media with the auxin and cytokinins shown in Figures 1 for 14 days before transfer to one of four media shown in Figure 2.

Thirty replicates were used for each of the second stages investigated, and the percentage organogenesis after 21 days in the second stage is shown in Figure 2.

1.2.1 Root Formation

1.2.1.1 Root formation was widespread from calluses generated on the various first stage media. There was no observable difference in the rhizogenic abilities of the four growth regulator interactions; all produced similar root regeneration frequencies at the respective auxin/cytokinin concentrations and their second stages. All growth regulator interactions, except high concentrations of auxin and cytokinin together, were capable of inducing high to very high (60-100%) rhizogenesis.

1.2.1.2 Of the four second stages tested, the hormone free media proved to be the most effective at promoting root formation, this was true irrespective of the first stage growth regulators. The inclusion of 10^{-7}M IAA reduced this ability only slightly, but did increase the amount of callusing during the second stage compared to that seen on tissue cultured on the hormone free medium.

Figures 2.1 - 2.4 Root and Shoot Regeneration from Hypocotyls Subjected to Various Two-Stage Culture Systems

Hypocotyls were cultured on twelve first stage medium described. After 14 days the callus was transferred to one of four second stages (see key).

Tissues were treated with the same cytokinin type in the second stage they were exposed to in the first. The data were recorded after 21 days in the second stage.

Tissue cultured on media containing 10^{-4}M 2,4-D or 10^{-4}M BAP in the first stage were not subcultured because no callus formation had taken place after the 14 days allotted to this stage.

Key:

Basal medium	MS supplemented with 2% sucrose
Replicates	20
Second Stage Media	1. no growth regulators
	2. 10^{-7}M cytokinin
	3. 10^{-7}M IAA
	4. 10^{-7}M cytokinin/10^{-7}M IAA

roots - 

shoots - 

Figure 2.1 Organogenesis from a Two-Stage System Utilising 2,4-D and BAP.

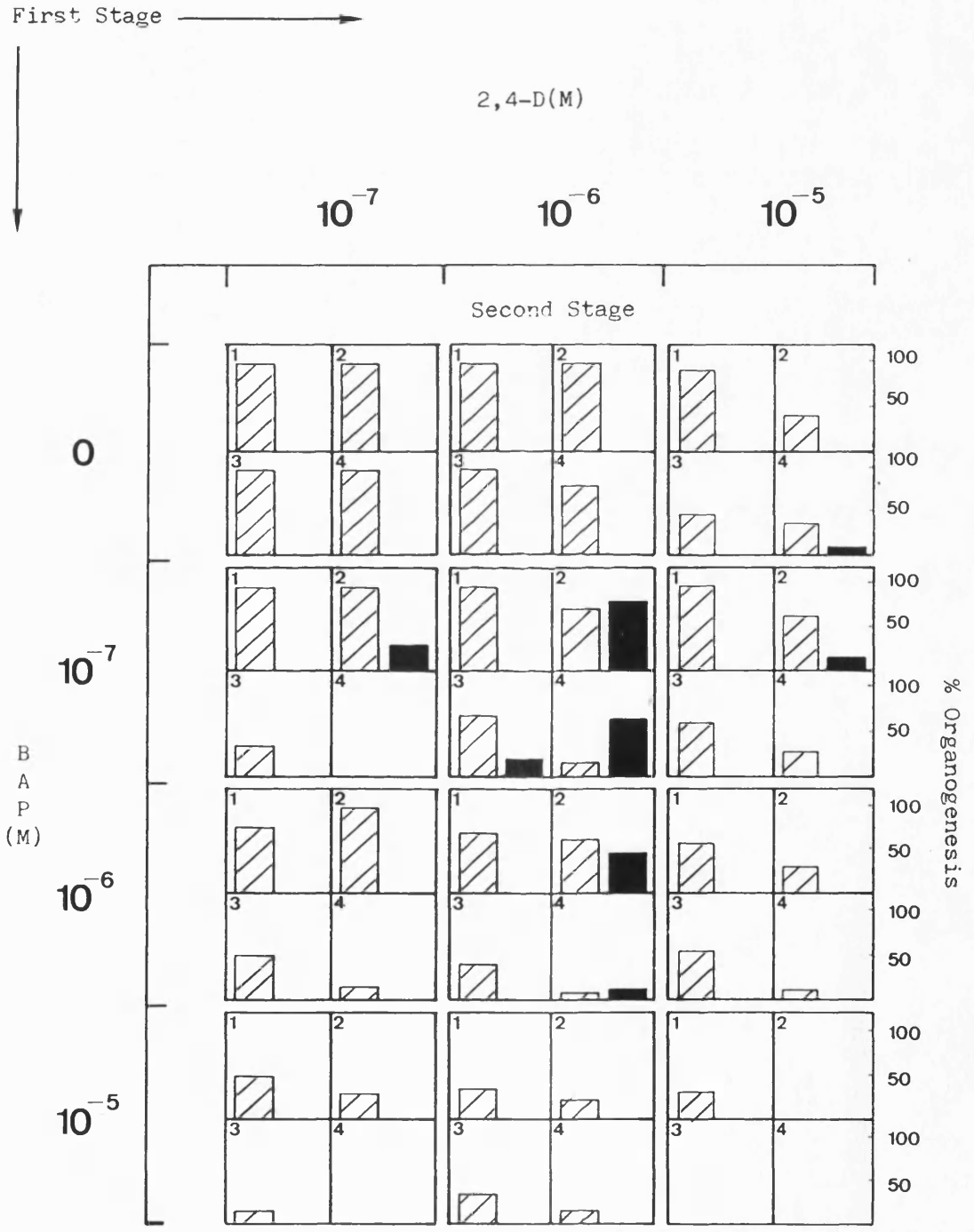


Figure 2.2 Organogenesis from a Two-Stage System Utilising 2,4-D and Kinetin.

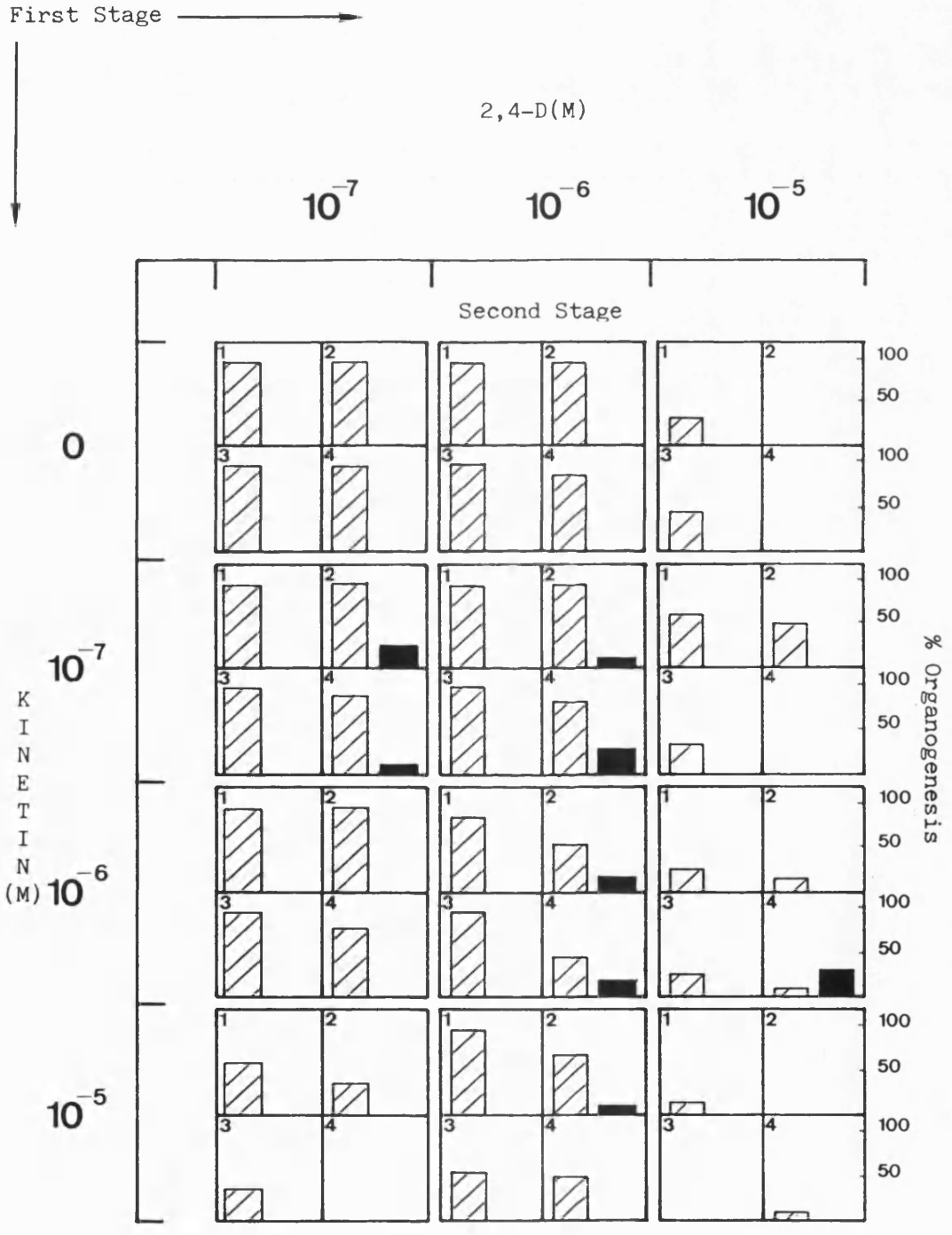


Figure 2.3 Organogenesis from a Two-Stage System Utilising NAA and BAP.

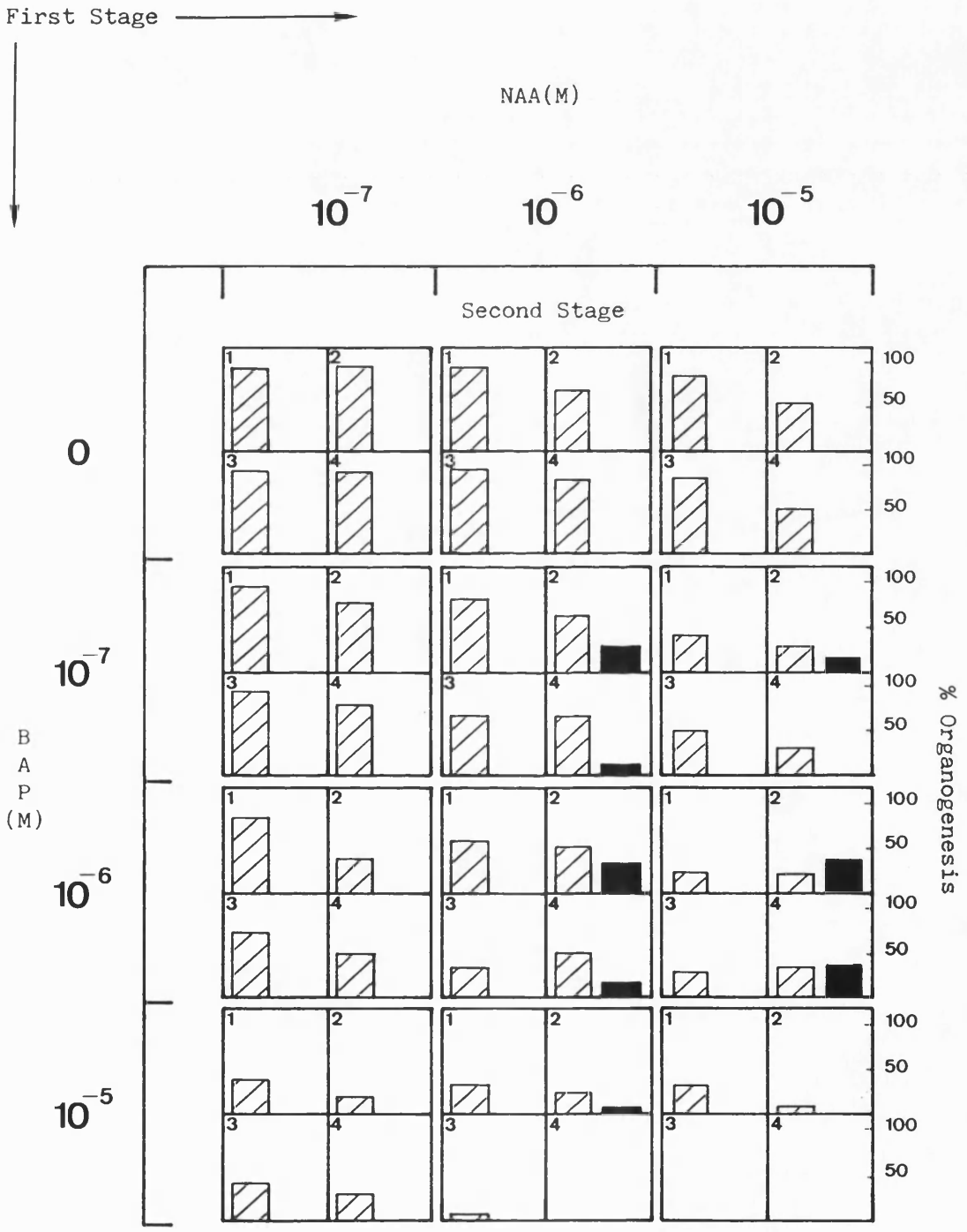
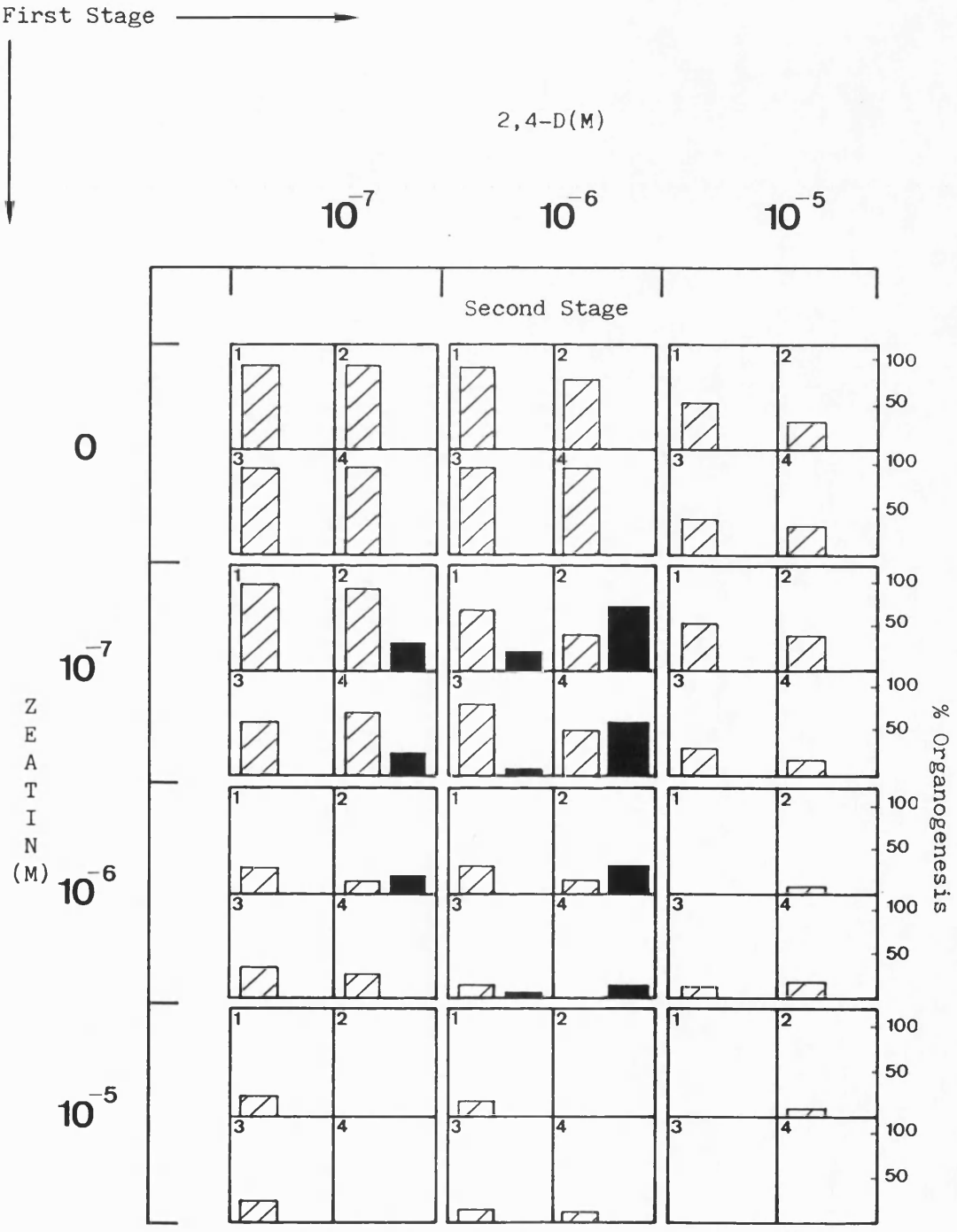


Figure 2.4 Organogenesis from a Two-Stage System Utilising 2,4-D and Zeatin



When the Stage 2 medium was supplemented with cytokinin, rhizogenesis was reduced; this was observed from all the first stages, but especially when BAP or zeatin was the cytokinin employed, kinetin was, overall, less effective at reducing root formation. Supplying IAA in addition to the cytokinin had little, or no, effect on rhizogenesis, but did promote the greatest degree of callus formation in the second stage (results not shown).

1.2.2 Shoot Formation

As in the one-stage system (see Section 1.1) caulogenesis was a less frequent event than rhizogenesis and for high frequency shoot formation a more specific first and second stage culture system was required than for root production. Differences were also observed between the four growth regulator interactions and the four second stage media with regards to their ability to generate callus capable of shoot formation, and then to induce that organogenic event.

Of the four first stages, 2,4-D/BAP and 2,4-D/zeatin were considerably the most effective at producing callus competent for caulogenesis. Replacing either of these with kinetin reduced the percentage shoot formation, and while NAA induced caulogenic callus in a relatively wide range of first stage auxin/cytokinin combinations, it was not as effective as 2,4-D at promoting high frequency regeneration.

The range of first stages that could generate caulogenically competent callus was considerably smaller than that for rhizogenesis. Only basal medium supplemented with 10^{-7}M auxin and 10^{-7}M or 10^{-6}M cytokinin was able to generate tissue capable of shoot formation.

In the second stage, inclusion of a cytokinin was necessary to induce shoot regeneration. Media containing no growth regulator supplements, or IAA on its own, were ineffective at promoting shoot production, while adddition of low levels of IAA with the cytokinin did not promote the frequency of shoot formation over that of cytokinin alone.

As in the one-stage system BAP and zeatin were equally superior to kinetin at inducing shoot regeneration, with the highest percentage of calluses undergoing caulogenesis when a first stage of 14 days duration on 10^{-6}M 2,4-D/10^{-7}M BAP or zeatin was followed by transfer to 10^{-6}M of the respective cytokinin. The values obtained here, approximately 80%, were greater than those achieved when a one stage system was employed (see Section 1.1).

2. Development of a Regeneration System for the Genotype Columbia from Various Media Supplemented with 2,4-D and BAP

The experiments described in Section 1 screened a range of growth regulators and their interactions and demonstrated that a combination of the auxin 2,4-D and the cytokinins BAP and zeatin used in a two-stage culture system, at low to medium concentrations (10^{-7}M-10^{-6}M), was the most effective regime for inducing high frequency, de novo, shoot regeneration in Arabidopsis thaliana. It was decided that this system should be examined in greater detail utilising material of known genetic background, especially Columbia, the genotype most commonly used as a laboratory test plant. The cytokinin BAP was favoured for these further studies as despite similar organogenic abilities (see Section 1) the former is considerably more cost effective.

2.1 Response of Hypocotyls of the Genotype Columbia to a One-Stage Culture System Utilising 2,4-D and BAP

Hypocotyls of the genotype Columbia were excised and cultured in 10 x 10 cm square repli-dishes, with the basal medium in each well containing a different concentration of 2,4-D and BAP. The range of responses to the various concentrations and interactions of the growth regulators is shown in Figure 3.1.

After 28 days in culture a number of distinct reactions similar to the patterns described in Section 1, were observed:

2.1.1 No Observable Reaction

BAP alone, at all concentrations, and 2,4-D at very high concentration (10^{-4}M) produced no visible development of the hypocotyls. These remained exactly as at the time of explanting except in the case of 10^{-4}M 2,4-D and 10^{-4}M BAP where the tissue became necrotic after about fourteen days.

2.1.2 Callus Formation

Callus formation was the most common response observed with four distinct types being produced.

2.1.2.1 Combinations of 2,4-D and BAP at low to medium concentrations (10^{-7}M to 10^{-6}M) produced the greatest degree of callus formation in hypocotyl explants. All of these were healthy, green and compact, with the exact amount of callusing dependant of the specific amount of each hormone, see Figure 3.1.

2.1.2.2 Increased growth regulator concentrations reduced the vigour of callus production. These became increasingly smaller wetter and paler in colour towards the combination 10^{-5}M 2,4-D/10^{-5}M BAP where the tissue was very small and orange in colour with an altogether unhealthy appearance.

2.1.2.3 2,4-D used alone at concentrations above 10^{-7}M induced small, wet, knobbly, white callus. The tissue on 10^{-6}M 2,4-D was vigorous with numerous root-like projections and asssociated root hairs: these often developed to form proper roots after about 14

Figures 3.1 - 3.2 Callus Formation and Organogenesis from Hypocotyls and Cotyledons of the Genotype Columbia after 28 Days Culture on Media Supplemented with Various Concentrations Of 2,4-D and BAP

Hypocotyls and cotyledons were cultured in 10 x 10cm square petri dishes for 28 days with the concentrations 2,4-D and BAP as shown. The cultures were scored after 28 days for the relative size of the callus and any organogenesis.

Key:

Basal medium	MS supplemented with 2% sucrose
Replicates	40
Callus size	* = minimum to ***** = maximum

callus -  frequency and degree of response

roots - 

shoots - 

Figure 3.1 Response of Hypocotyls to 28 Days Culture on Various Concentrations of 2,4-D and BAP.

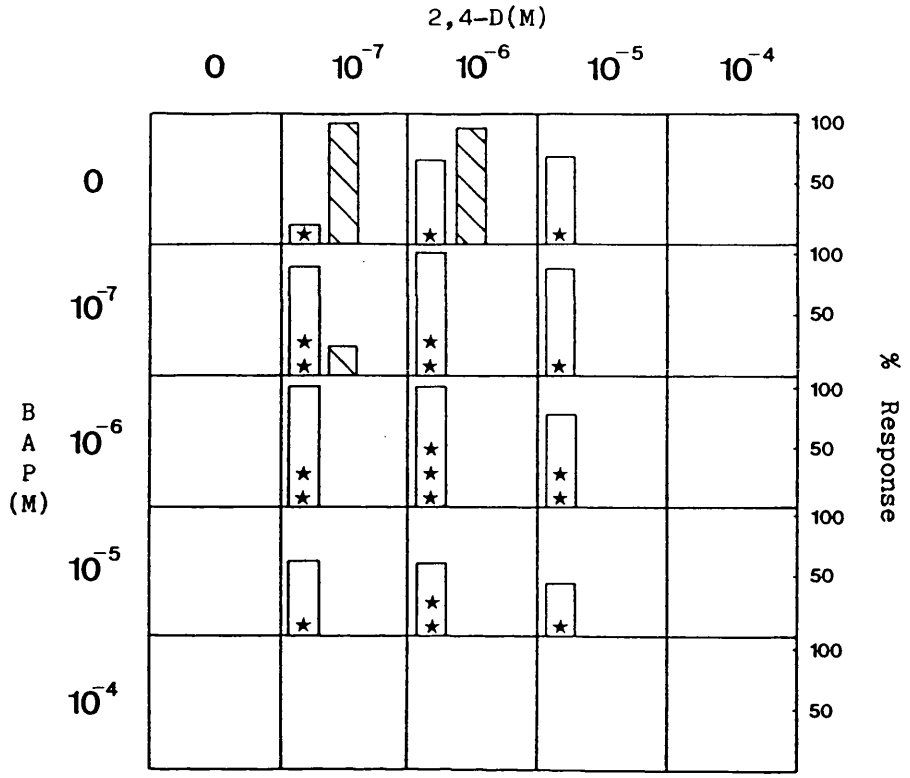
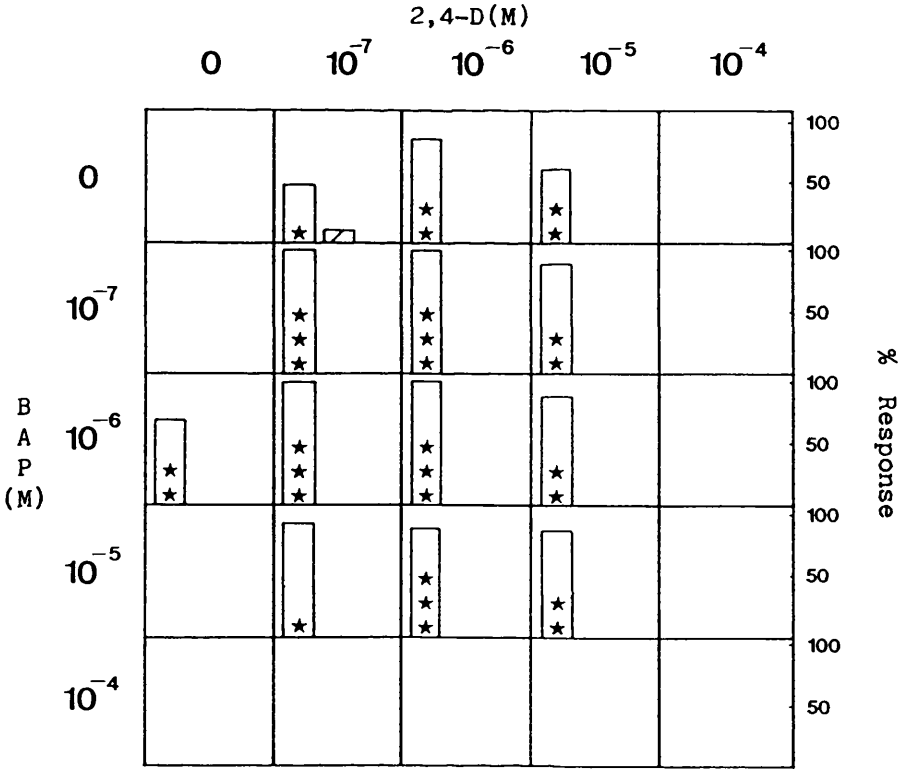


Figure 3.2 Response of Cotyledons to 28 Days Culture on Various Concentrations of 2,4-D and BAP.



days. The hairless callus formed on 10^{-5}M 2,4-D was less vigorous than that formed on 10^{-6}M, and it possessed no organised structures. Instead this tissue developed by transverse swelling along its whole length to form a rough, string-like callus like that described in Section 1.1.2.4.

2.1.2.4 The specific interaction 10^{-6}M 2,4-D/10^{-7}M BAP caused the formation of a highly distinctive callus type. Hypocotyls placed on this medium developed to form a green, healthy, compact callus with no visible organisation and little, if any, hair formation. Initially this tissue was not dissimilar to that produced by other callus-forming media, but after 16 to 20 days a white, smooth, undulating tissue developed on the upper surface of the older, greener callus. This phenomenon was not seen with any other growth regulator regime and was observed on only a proportion of 10^{-6}M/10^{-7}M examples.

The smooth, undulating tissue appeared to very similar to that described as embryogenic by other workers, and therefore callus generated under these culture conditions was considered to be potentially embryogenic (PE) (see Plate 1). It was studied separately, and in detail, as a possible starting point for the induction of somatic embryogenesis in Arabidopsis thaliana (see Section 6).

2.1.3 Root Formation

Roots were formed in three of the twenty five hormone regimes investigated.

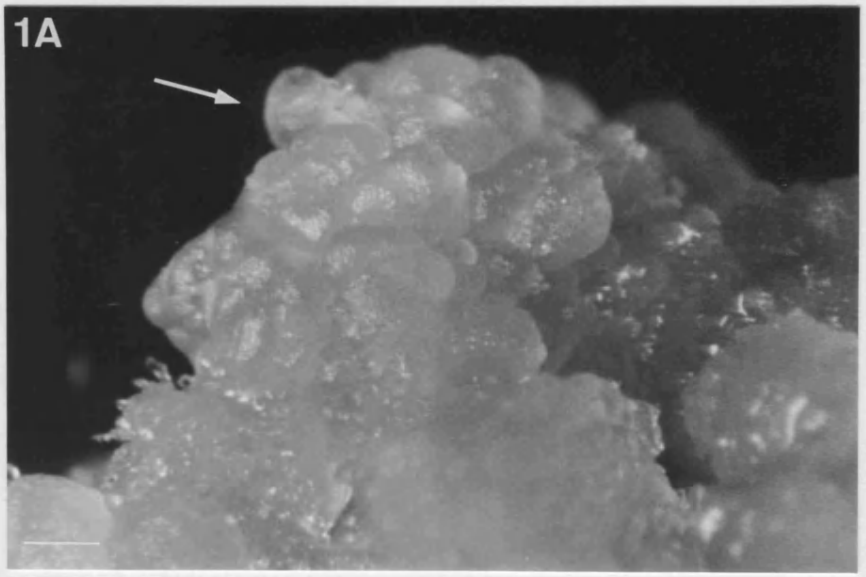
Plates 1A and 1B Tissue Identified as Potentially Embryogenic Formed from Hypocotyls of the Genotype Columbia after 21 Days on the First Stage Medium

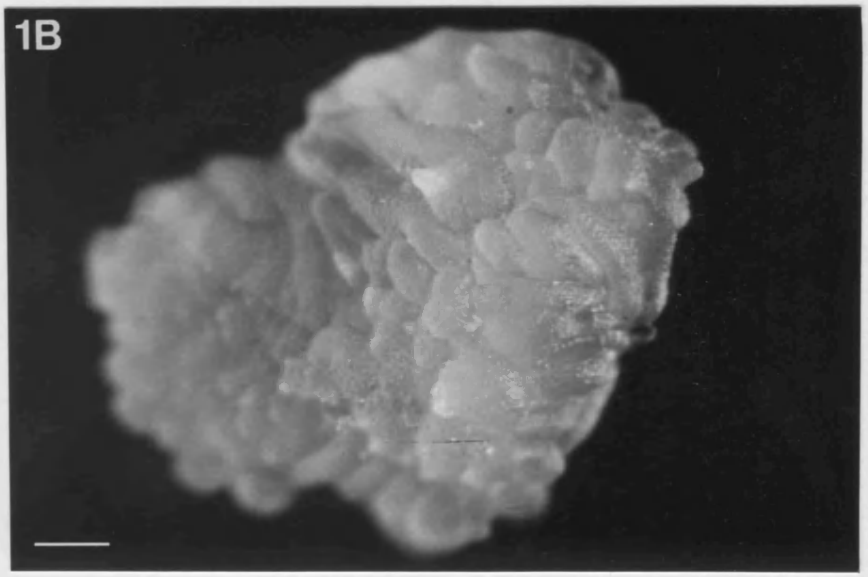
Plate 1A The callus surface is covered in a mixture of tissue types, but arrowed are areas with a pale colour, apparently smooth surface and convoluted shape. This is the PE tissue.

Scale bar = 200μm

Plate 1B Similar tissue shown at higher magnification to show its organised nature and the smooth and undulating surface characteristics.

Scale bar = 100μm


1A


1B

As reported in Section 2.1.2.3 hypocotyls cultured on 10^{-6}M 2,4-D produced a "hairy" callus which formed roots after 16 to 21 days. Roots, however, were formed within 14 days, and in greater amounts, when the explants were placed on 10^{-7}M 2,4-D: in this case no callus formation was observed prior to rhizogenesis, but it was often seen subsequently on the newly produced roots.

Root regeneration also took place from tissue cultured on 10^{-7}M 2,4-D/10^{-7}M BAP, but this was of low frequency, (c. 20%) and was preceeded by considerable callus formation.

2.1.4 Shoot Formation

Unlike the undefined genotype studied in Section 1 no shoots regenerated within the observation period from the calluss tissue formed by hypocotyls of Columbia in this one-stage culture system.

2.2 Response of Cotyledons of the Genotype Columbia to the One-Stage Culture System Utilising 2,4-D and BAP

Cotyledons were excised and subjected to the same culture procedure and growth regulator interactions as the hypocotyls.

Like the hypocotyls, cotyledons produced callus at all hormone concentrations, except when either was present at 10^{-4}M or when 2,4-D was not included in the basal medium. An exception to this was zero 2,4-D/10^{-6}M BAP where a reasonably sized, very pale green callus was formed (see Figure 3.2). This was the only instance of callus formation in the absence of 2,4-D.

Compared to hypocotyls, the cotyledons tended to produce larger calluses at all the respective hormone interactions. These were green, dry and healthy but as with the hypocotyls, colour and size diminished as the growth regulator concentrations were increased.

10^{-7}M 2,4-D was the only medium in which organised structures were seen. This tissue was almost white in colour and possessed numerous root-like projections many of which were hairy. Although few true roots were observed this tissue was similar in all other respects to that produced by hypocotyls cultured on 10^{-5}M 2,4-D.

No shoots were produced by the callus generated from cotyledon explants.

2.3 Response of Hypocotyls of the Genotype Columbia to a Two-Stage Culture System Utilising 2,4-D and BAP.

The response of hypocotyls to a two stage culture system was studied by subculturing tissue formed by the twelve callus-inducing first stage media shown in Figure 3 and described above (Section 2.2) onto a number of different second stages. In addition the explants were cultured for one, two or three weeks before transfer in order to investigate the effect of different first-stage durations on organogenesis. The second stage media contained various concentrations of BAP. This cytokinin was chosen because it had been shown to be more effective at inducing shoot regeneration than kinetin (see Section 1) and was less expensive to

use than zeatin. Twenty replicates were used in each of the second stages and the response after twenty one days in Stage 2 is shown in Figure 4.

Hypocotyls cultured for longer than one week on 10^{-7}M 2,4-D, or two weeks on 10^{-6}M 2,4-D were not transferred as they had regenerated roots within this time. It was considered that subculture of such tissue would, in effect, constitute treatment of the new organ type and not that of the hypocotyl. The original organ type was assessed to have been lost as a result of the rhizogenic event.

The calluses responded in one of three ways to culture in the various second stages. These were, root and/or shoot formation or callus growth without regeneration.

2.3.1 Callus Production and Growth

Continuous growth without organogenesis was the single most common reaction seen after three weeks culture in the second stage.

High concentrations of 2,4-D and BAP, especially when in combination in the first stage, promoted callus production rather than organ formation. Even with media which induced organogenesis a prolonged culture in the first stage promoted disorganised development at the expense of regenerated structures.

The callus formed in the second stage was of two main types. High 2,4-D and BAP concentrations (10^{-5}M) in Stage 1 produced the small, wet callus as described in Section 2.1.2.2. On transfer and culture in the second stage this tissue acquired a

Figure 4 Organogenesis from Hypocotyls of the Genotype Columbia Subjected to a Two-Stage Culture System with a First Stage of Varying Duration

Hypocotyls were cultured on the 12 callus inducing combinations of 2,4-D and BAP shown in Figure 3.1 for one, two or three weeks before transfer to one of four second stages with a range of BAP concentrations from zero to 10^{-5}M.

Specimens cultured on 10^{-7}M 2,4-D in the first stage for two weeks or longer and 10^{-6}M 2,4-D for three weeks were not subcultured because by this time these explants had regenerated roots.

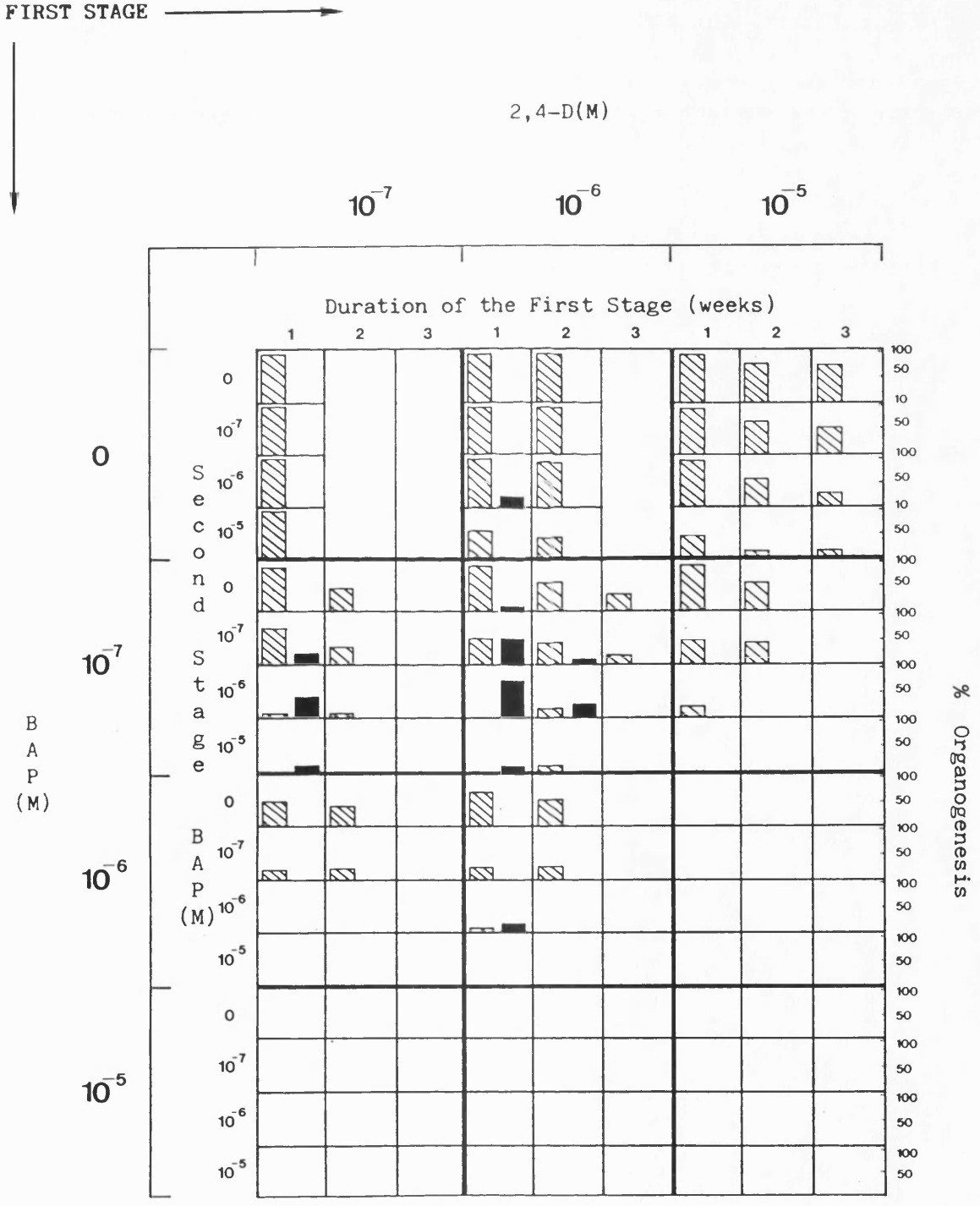
Key:

Basal medium	MS supplemented with 2% sucrose
Replicates	40

roots - 

shoots - 

Figure 4 Organogenesis from Hypocotyls of the Genotype Columbia Subjected to a Two-Stage Culture System with a First Stage of Varying Duration



progressively yellowish/brown unhealthy appearance. This was apparent at all BAP concentrations but was most marked when the second stage cytokinin concentration was also high.

Low to medium growth regulator concentrations (10^{-7}M to 10^{-6}M) induced a much healthier, greener callus type which continued to grow vigourously throughout the culture period. The specific interactions which facilitated organogenesis are stipulated below, but in many cases no regenerants were obtained. In these the callus that was formed varied with the growth regulator regime to which the tissue was subjected.

Root-like projections were common on many of the calluses and although this was most often associated with hair formation, sometimes in copious amounts, these never developed to form the organ proper. This response was to be found most often in tissue which had been exposed to high 2,4-D/BAP in the first stage, and low BAP in the second.

The development of the PE tissue in numerous second stage media is examined in Section 5.

2.3.2 Root Formation

Root formation was seen to vary inversely with the concentration of BAP present in the medium, especially that of the first stage. Rhizogenesis was widespread when BAP was omitted, or present in low concentration. In Stage 1 increasing the level of this cytokinin caused reduction in the number of calluses capable

of root formation. Rhizogenesis was prolific from callus cultured on 2,4-D alone in the first stage, regardless of the culture duration or the cytokinin concentration in the second stage.

2.3.3 Shoot Formation

Figure 4 shows that, although shoots can be obtained from a number of first and second stage sequences, high frequency caulogenesis is only associated with two first stage media. Only 10^{-7}M 2,4-D/10^{-7}M BAP and 10^{-6}M 2,4-D/10^{-7}M BAP were effective at inducing a large percentage of the calluses to form shoots, and then, only when the tissue was subcultured after one week in Stage 1. Continued culture in the first stage rapidly reduced the tissue's ability to respond to the shoot-inducing second stage medium.

The requirements for successful caulogenesis would appear to be much stricter than for rhizogenesis. The latter was achieved at high frequency from six different first stages and was reduced in frequency only slightly by prolonged first stage culture. Successful shoot formation on the other hand was obtained from only two of the first stages investigated, was limited by the concentration of cytokinin in the second stage and, more severely, by the time spent in the callus induction medium.

Of the four second stage BAP concentrations tested, 10^{-6}M was shown to be the most effective at inducing high frequency shoot formation. This is confirmed by Figure 5, which is constructed

from data collected from 100 replicates subjected to the two caulogenic first stages (10^{-7}M 2,4-D/10^{-7}M BAP and 10^{-6}M 2,4-D/10^{-7}M BAP, see Figure 4) and the various second stages.

This experiment was carried out with 100 replicates in order to allow statistical comparison between the effectiveness of the different first and second stages. It indicates that the optimal procedure for de novo shoot formation from hypocotyls of Columbia was a first stage of one week's duration on a medium containing 10^{-6}M 2,4-D/10^{-7}M BAP, followed by transfer to, and culture in, a medium of 10^{-6}M BAP. This was significantly the more effective first stage (at P=0.01 when comparing the 10^{-6}M second stage responses) and induced more calluses to undergo caulogenesis than 10^{-7}M/10^{-7}M 2,4-D/BAP, at all respective second stage BAP concentrations.

In the second stage, some shoots were obtained on hormone-free medium but inclusion of BAP increased the frequency of regeneration many times to a maximum of around 70%. 10^{-5}M BAP was shown to be supra-optimal.

Although supplementation of the second stage with cytokinin increased caulogenesis, a penalty was paid in the quality of the regenerants obtained. The frequency of vitrification and malformation increased directly with increasing BAP concentrations. The severity of malformation tended to vary between individuals and in many cases transfer to a hormone free, activated charcoal medium allowed normal development to be re-established (results not shown). In other cases, including all regenerants recovered from

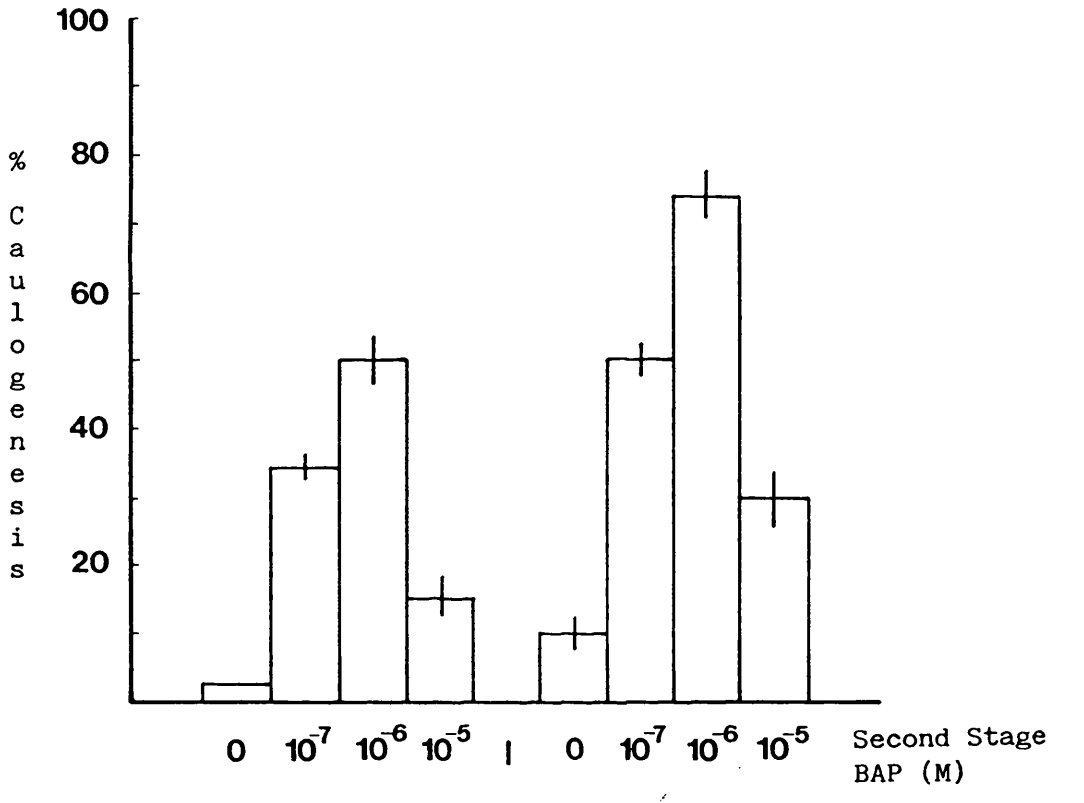
Figure 5 Comparison of the Caulogenic Effects on Hypocotyls of the Genotype Columbia of Two 2,4-D/BAP First Stage Regeneration Media when used in Combination with Different BAP Second Stages

The culture procedure described in Figure 4 was repeated with a larger number of replicates in order to study in more detail the caulogenic response of the two media identified as promoting shoot formation. The specimens were scored for shoot formation 21 days after transfer to one of the four second stage media.

Key:

Basal medium		MS supplemented with 2% sucrose
Replicates		100
First stage	-	10^{-7}M 2,4-D/10^{-7} BAP or
		10^{-6}M 2,4-D/10^{-7} BAP
Second stage	-	0, 10^{-7}, 10^{-6}M or 10^{-5}M BAP

Figure 5 Comparison of the Caulogenic Effects on Hypocotyls of the Genotype Columbia of Two 2,4-D/BAP First Stage Regeneration Media when used in Combination with Different BAP Second Stages



second stages containing 10^{-5}M BAP, the shoots were so disrupted as to make the "normalising" procedure impossible, and rooting and flowering unachievable.

3. Response of Various Genotypes and Explant Sources to Culture Systems Utilising 2,4-D and BAP

The experiments described in Section 2. determined the optimum procedure for the production of high frequency shoot regeneration from hypocotyls of Columbia. As more genotypes became available it was considered desirable to screen plants from a wider range of genetic backgrounds and explant types for their morphogenic potential under this system.

3.1 Comparison of the Callusing and Organogenic Reponse of Six Genotypes on Exposure to a One-Stage Culture System

Hypocotyls and cotyledons were excised and placed on 12 media supplemented with different concentrations and ratios of 2,4-D and BAP as shown in Figure 6. These media were chosen because they had been shown to induce callus formation in the two previous genotypes investigated (see Sections 1 and 2). Thirty explants from each genotype were cultured on each of these media, and the tissues scored for callus formation and organogenesis 28 days after explanting.

Figures 6.1 - 6.6 Callus Formation and Organogenesis from Hypocotyls and Cotyledons of Six Genotypes after 28 Days Culture on a Range of 2,4-D and BAP Media

Hypocotyls and cotyledons were placed on media supplemented various concentrations and combinations of 2,4-D and BAP. The results are shown for relative callus size, and root and shoot formation 28 days after explanting.

Key:

Basal medium		MS supplemented with 2% sucrose
Replicates		30
Callus size		* = minumum to ***** = maximum
callus	-	frequency and degree of response
roots	-	
shoots	-	

Figure 6.1 Response of the Genotype Columbia

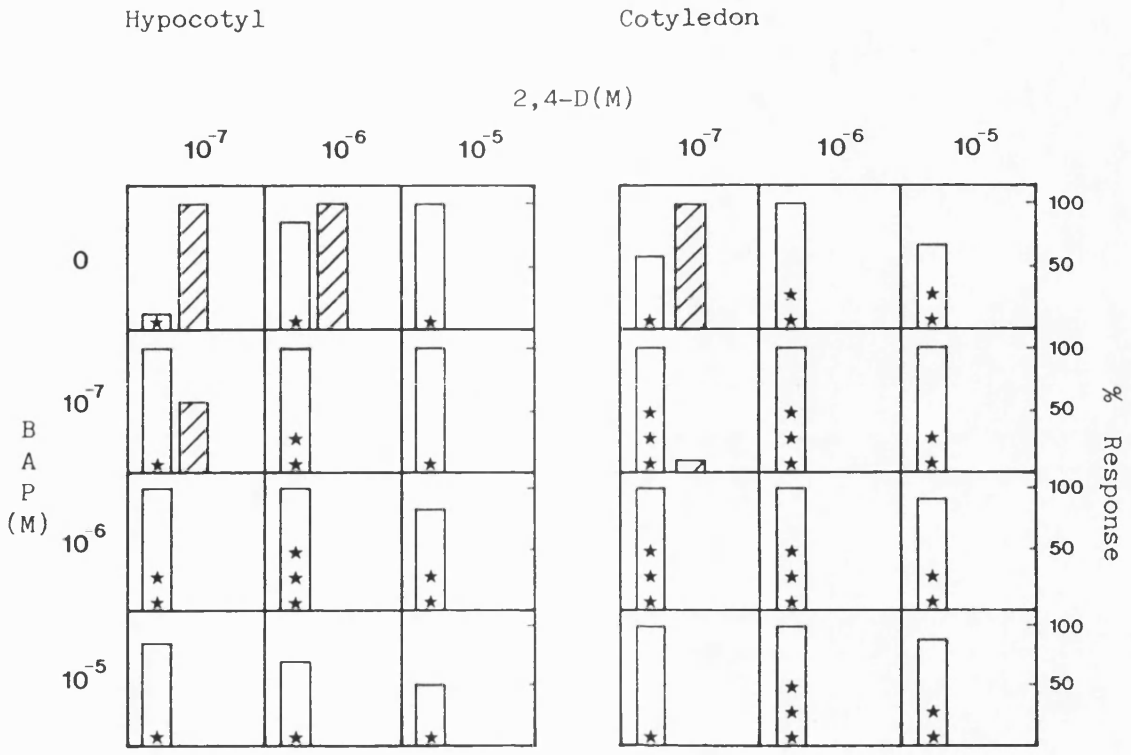


Figure 6.2 Response of the Genotype Estland

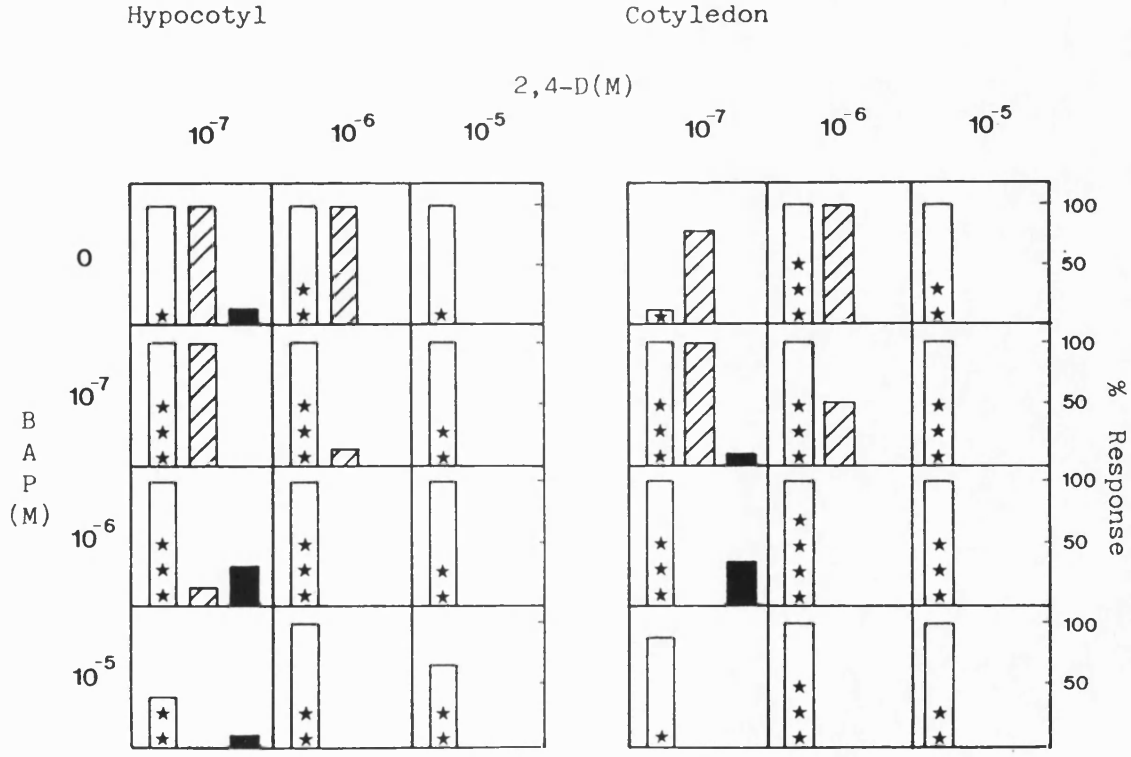


Figure 6.3 Response of the Genotype Landsberg

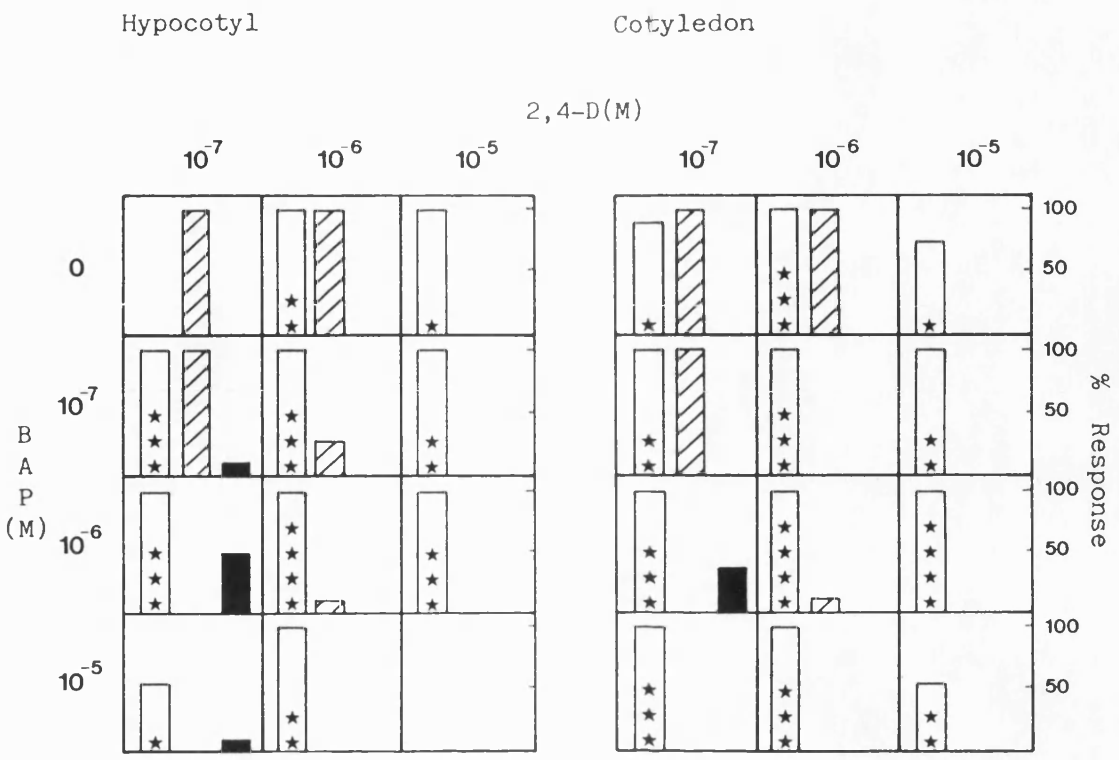


Figure 6.4 Response of the Genotype Dijon

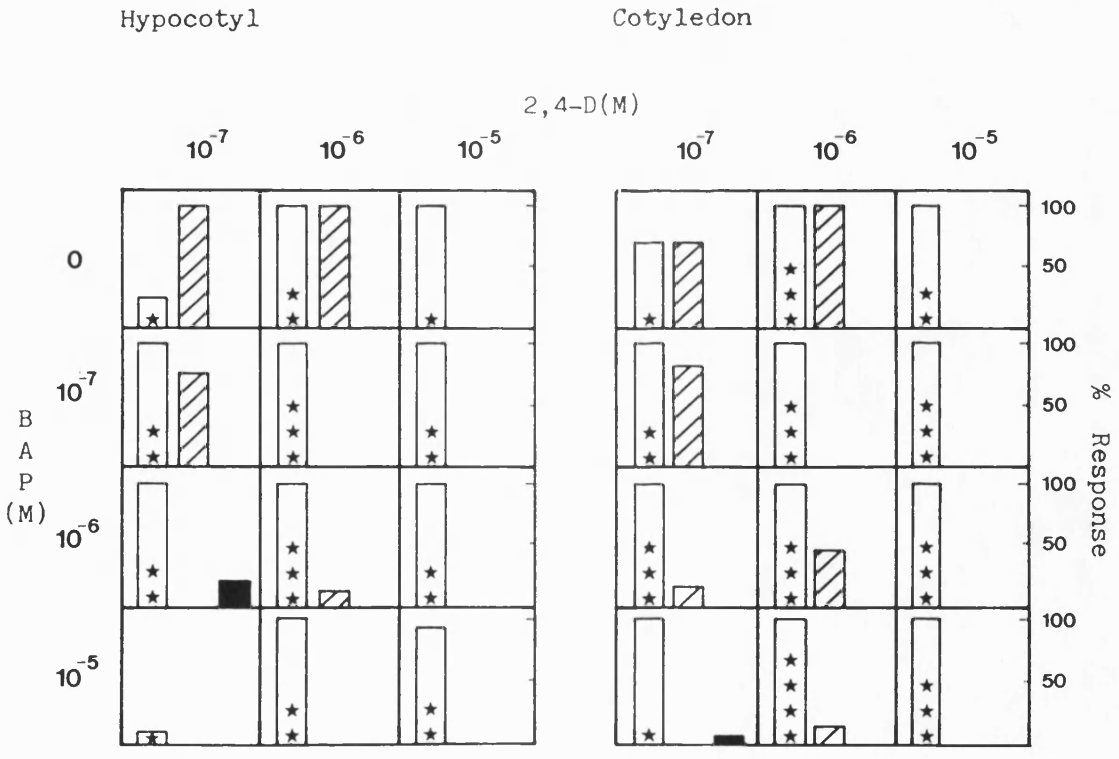


Figure 6.5 Response of the Genotype Benshiem

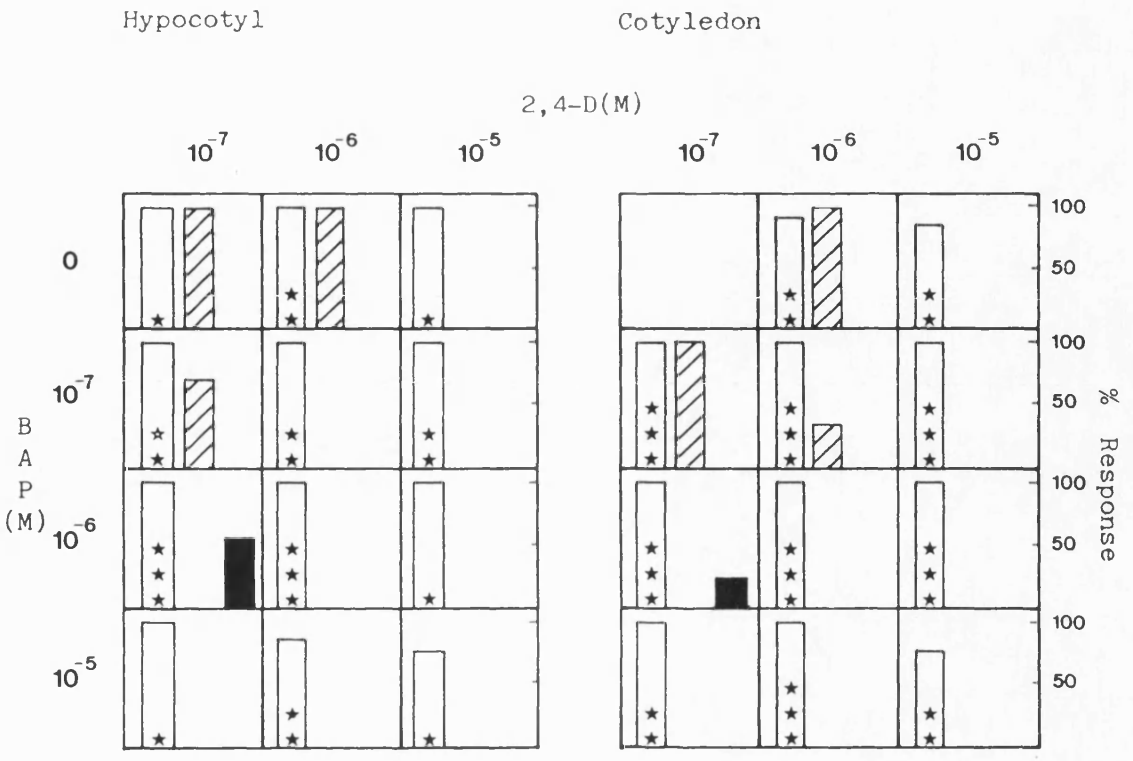
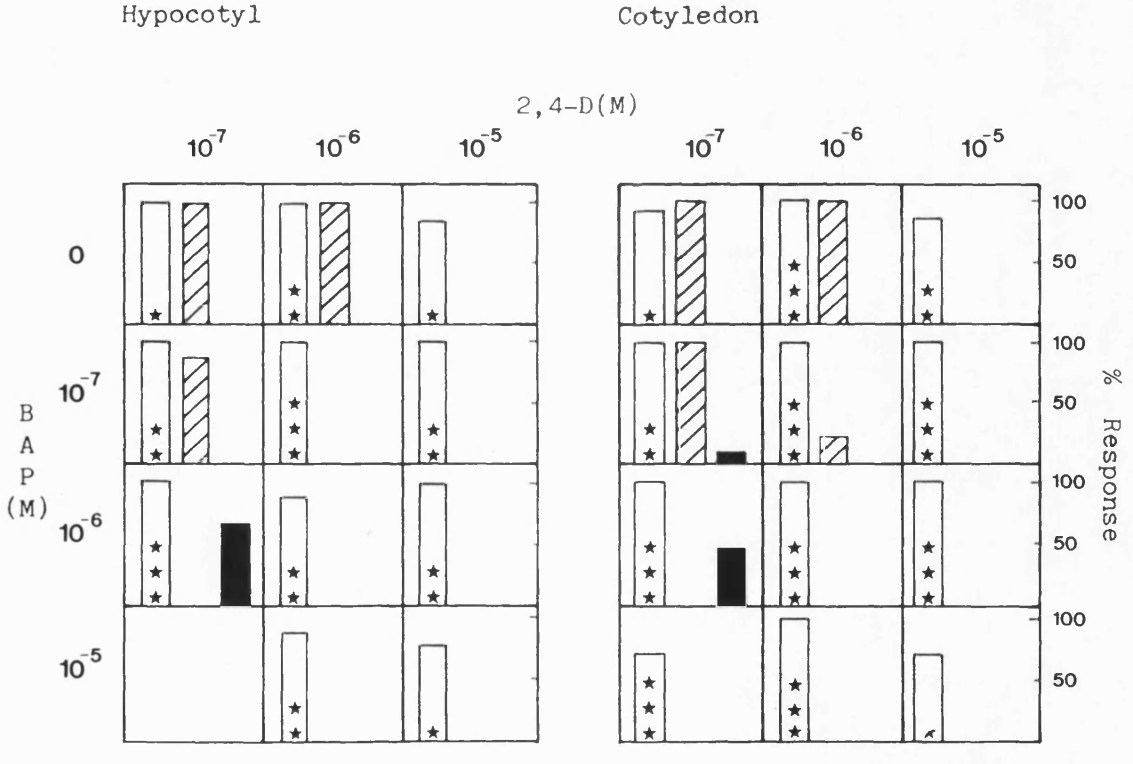


Figure 6.6 Response of the Genotype Enkeim



3.1.1 Callus Formation

The genotypes screened in this experiment responded in a similar manner to that described for Columbia in Section 2.1. although most formed larger calluses than the aforementioned: notably Dijon, Estland and Landsberg.

Callus production was greater from cotyledon explants than hypocotyls and was optimal when the medium was supplemented with auxins and cytokinins at low to medium concentrations (10^{-7}M-10^{-6}M); increasing this to 10^{-5}M reduced callusing.

3.1.2 Root Formation

High frequency root regeneration from tissue generated from the hypocotyls was restricted to three media: 2,4-D alone, at 10^{-7}M and 10^{-6}M and from callus produced on media supplemented with 10^{-7}M 2,4-D/10^{-7}M BAP. The same media were successful at regenerating roots from cotyledon-derived callus but, with this explant, rhizogenesis was also seen at low frequencies from other media, especially 10^{-6}M 2,4-D/10^{-7}M BAP.

3.1.3 Shoot Formation

Shoot formation was observed from all the genotypes except Columbia. This occurred from callus derived from both hypocotyls and cotyledons, but to a greater extent in the latter explant.

In the others high frequency caulogenesis was restricted to medium supplemented with 10^{-7}M 2,4-D/10^{-6}M BAP. The shoots appearing mostly between 15 and 25 days after explanting. Other media were incapable of inducing shoot formation, or could do so only at very low frequencies.

The percentage of calluses undergoing caulogenesis varied between the genotypes; Enkeim was the most productive with 65%, followed by Bensheim and Landsberg at 55% and 50% respectively, but was very low in Dijon and Columbia. The number of shoots produced by each regeneration event was low, in most cases between one and three per responding callus.

3.1.4 Formation of PE Tissue

The production of P.E. tissue (see Section 2.1.2.4) was rarely observed from the six genotypes screened in this experiment. PE tissue was formed only by callus growing on media supplemented with 10^{-6}M 2,4-D/10^{-7}M BAP and only by Columbia (70%), Enkeim (30%) and Estland (10%).

3.2 Response of Various Genotypes and Explant Sources to the Two-Stage Culture System Devised for Maximum Caulogenesis in Columbia

In order to produce a range of explant types seeds were sown on MS agar medium and germinated axenically. This took place within two days, and thereafter organs from the six genotypes were

excised and explanted as they became available from the developing plantlet. After four days considerable elongation of the hypocoyls necessitated that they be cut into three equal segments. These were termed as 1, 2 and 3 in basipetal order, and cultured as separate explant sub-types.

All explants were subjected to the culture system devised for hypocotyls of Columbia: 10^{-6}M 2,4-D/10^{-7}M BAP for seven days followed by transfer to 10^{-6}M BAP. Thirty replicates were used for each treatment. As the culture system was constant it was possible to examine the effects of explant type, age of the mother plant and genotype on shoot and root regeneration. The results are displayed in Figure 7.

3.2.1 Effect of Mother Plant Age

The influence of mother plant age can be examined by comparing the organogenic ability of the hypocotyl segments, cotyledons and first leaves. The effect of age on the petioles and second leaf is not clear due to the shorter time span that could be studied in these ontogenetically more mature organs.

The data obtained from the six genotypes studied indicate that, over the time period examined, the age of the mother plant ie. the age of the tissue at explanting, had an effect on regenerative ability, although the extent of this varied with the type of explant and genotype studied.

Figures 7.1 - 7.6 Organogenesis from Explants of Various Types and Ages from a Two-Stage Procedure with a Number of Genotypes

Explants were excised from axenically grown plantlets at various times and exposed to the two-stage culture system developed for maximum caulogenesis from hypocotyls (see Section 2.3). Subculture took place after one week on the first stage. The frequency of organogenesis was scored after 21 days in the second stage.

Key:

Basal medium	MS supplemented with 2% sucrose
Replicates	20
First stage	10^{-6}M 2,4-D/10^{-7}M BAP
Second stage	10^{-6}M BAP

Explants;

- Hyp.1-3 = segments of the hypocotyl 1 to 3 (basipetal order)
- Cot. = the cotyledon
- C.Pet. = the petiole of the cotyledon
- Lf.1 = the first leaf
- Pet.1 = the petiole of the first leaf
- Lf.2 = the second leaf
- Pet.2 = the petiole of the second leaf

root = ⌐¬

shoot = ■

Figure 7.1 Organogenesis from Explants of the Genotype Columbia

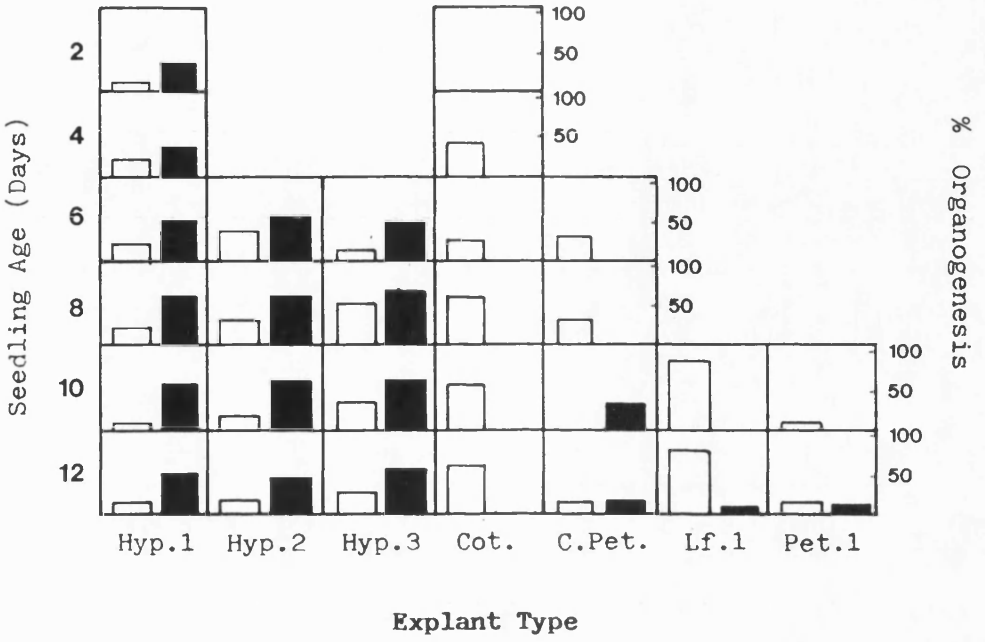


Figure 7.2 Organogenesis from Explants of the Genotype Estland

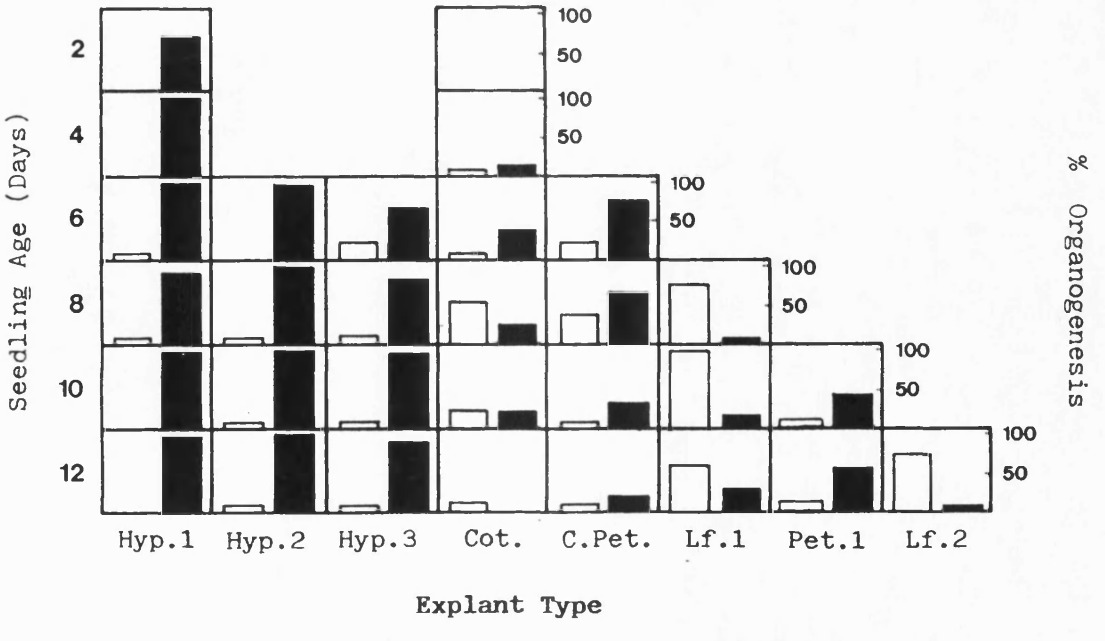


Figure 7.3 Organogenesis from Explants of the Genotype Landsberg

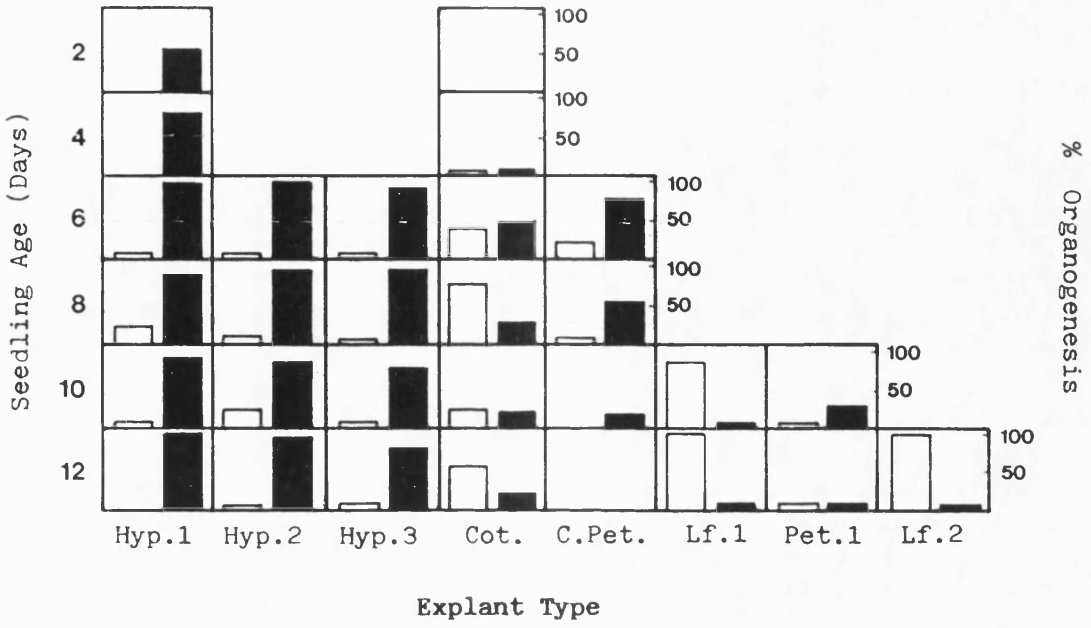


Figure 7.4 Organogenesis from Explants of the Genotype Dijon

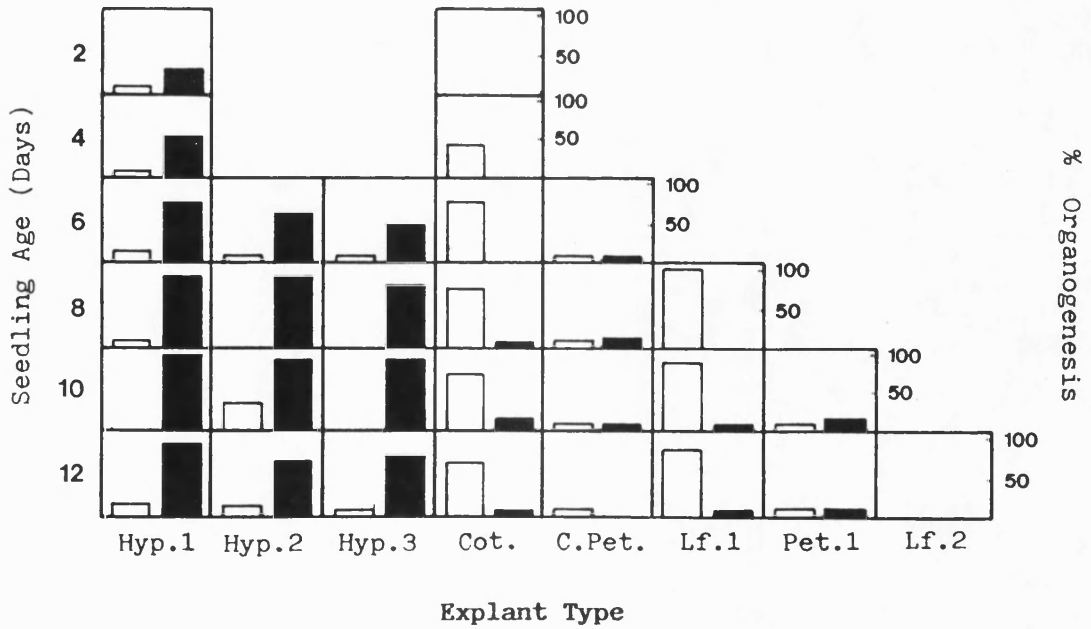


Figure 7.5 Organogenesis from Explants of the Genotype Bensheim

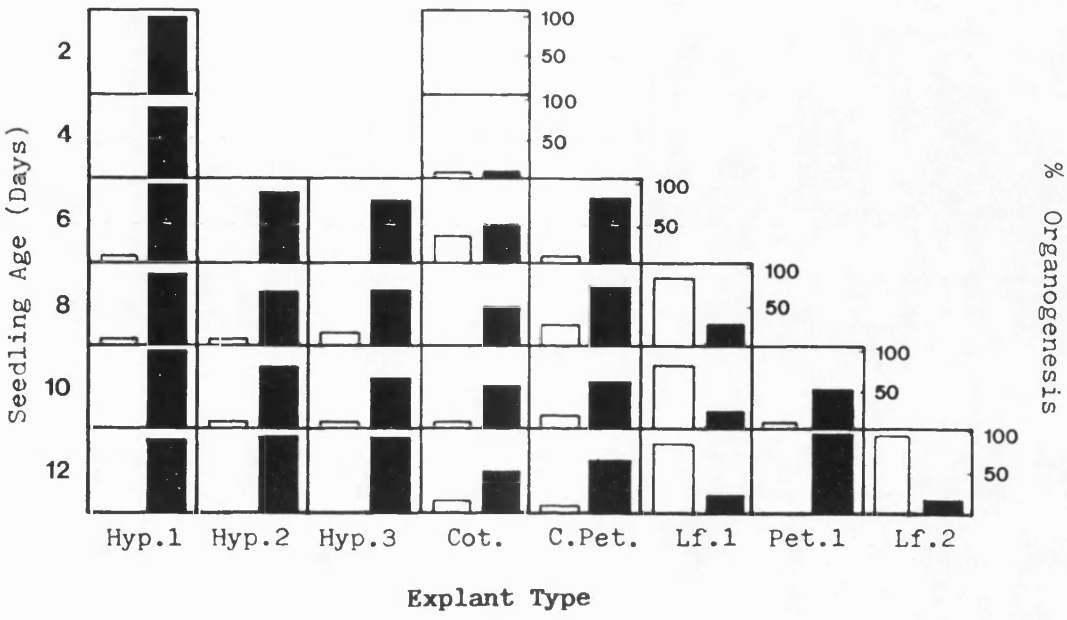
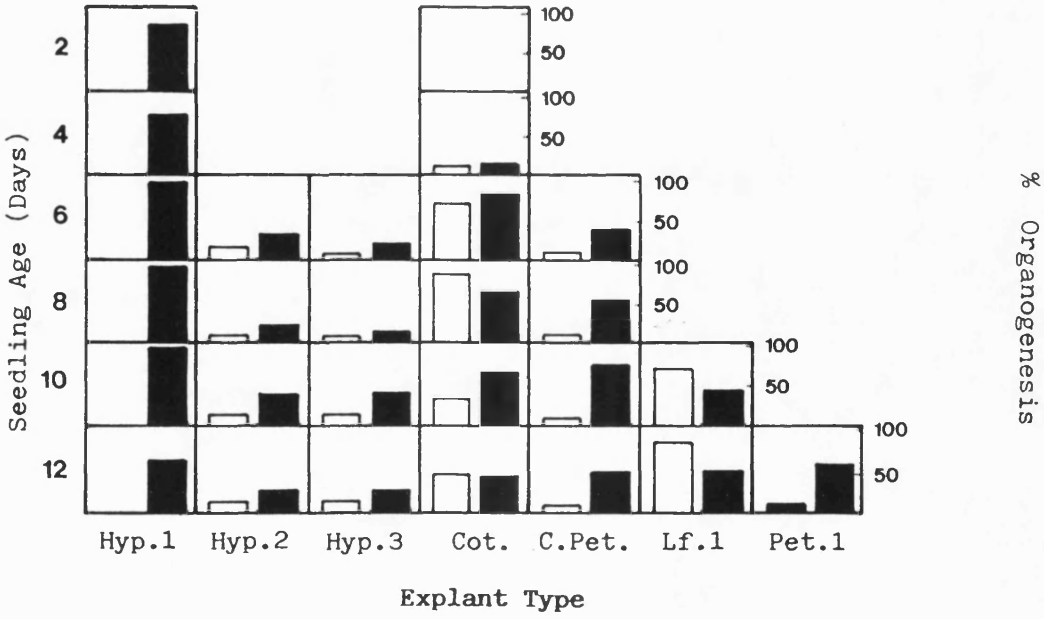


Figure 7.6 Organogenesis from Explants of the Genotype Enkeim



In hypocotyls there was little, or no difference in potential across the age span except in the youngest tissue of the genotypes Dijon and Landsberg, and in the oldest tissue in Enkeim. Here shoot production was below the high frequency optimum achieved by intermediately aged hypocotyls.

Considerable greater age effects were obtained for cotyledons. This organ did not achieve competence for organogenesis until four days after germination, and in all the genotypes caulogenic competence was maximal after germination plus six days. Thereafter the number of reacting explants decreased with the increasing age of the tissue.

Root production did not appear to follow any such pattern, but, as for shoots, it did not occur until the cotyledons were at least four days after germination.

With regard to the first leaf the data is less clear: in Landsberg, Estland and Bensheim caulogenic competence was greatest when the tissue was at its youngest explantable age and this capacity was greatly reduced in older first leaves. In Columbia the first leaf did not aquire the ability to form shoots until much later, while in Enkeim and Bensheim there was little loss of potential with increasing age.

3.2.2 Genotypic Effects

Enkeim was the most responsive genotype tested; all of its explants, except the hypocotyl sub-types 2 and 3 responded well to this culture regime, forming shoots at high, to very high, frequency. Hypocotyls consistently regenerated shoots at

frequencies of 90 - 100%, while cotyledons, petioles and leaves, which were commonly recalcitrant in other genotypes, formed shoots at frequencies of 70% and above.

The poorest genetic source was that of Columbia. This was the least reactive, regardless of the age or type of tissue tested. Its optimal caulogenic response was only c.65% from hypocotyls, whilst almost no regeneration was obtained from its cotyledons or other tissues. Notably, Columbia was particularly rhizogenic producing roots at high frequency (30 - 50%) under a culture system designed to promote de novo shoot formation.

The remaining genotypes responded to varying extents between the high and low values of Enkeim and Columbia. Of note is Dijon; here callus derived from the hypocotyl was capable of high frequency shoot formation, but the other explant tissues failed to respond, or did so at very low percentages.

3.2.3 The Effect of Explant Type

As this regeneration system was primarily designed to induce high frequency regeneration from hypocotyls this organ was predictably the most caulogenic explant source. In all, except Columbia, this explant type produced regeneration frequencies of 80 - 100%, with the reacting calluses producing multiple shoots.

Only in Enkeim does the position of the hypocotyl segment appear to be critical. In this otherwise caulogenic genotype, the explant sub-types 2 and 3 had a significantly ($p=0.01$) lower shoot forming ability than the region immediately below the apical meristem (see Figure 7.6). The reason for this is not known, but

in Enkeim hypocotyl extension was considerably greater than in the other genotypes. As a result these were cut into five parts with only the uppermost, middle and lowermost sections being cultured. It is possible that this elongation is responsible for the reduction of shoot forming ability, certainly the spacial separation from the apical meristem to the middle and lower sections was greater in this genotype, and these explant sub-types did callus to a lesser extent than the uppermost region (results not shown).

The remaining organs can be split into two types; foliose (cotyledons and leaves) and petiole structures. Figure 7 shows clearly that the organogenic potential of the two groups varies considerably under these culture conditions. Foliose organs had poor shoot forming, but good root forming abilities, while the petioles were highly caulogenic in all of the genotypes.

This culture system was capable of inducing shoot regeneration from all the explant types tested, but for foliose structures at frequencies below those obtained for hypocotyls and petioles.

In summary, the results displayed in Figure 7 indicate five points:

1. A large number of explant types were capable of forming callus, and regenerating shoots from this callus, although the extent of this ability varied with the genotype in question.

2. The formation of roots and shoots appeared to be mutually exclusive. Tissue types which produced a high frequency of roots, produced few shoots and vice versa.

3. All six of the genotypes were highly caulogenic under this culture regime. In fact Columbia, the type for which the system was initally designed, was consistantly the poorest, tending instead towards rhizogenesis.

4. Age of the mother plant at the time of explanting had limited bearing on the regenerative potential of the tissues over the time period examined here.

5. The position from which the hypocotyl segment was taken had an effect on the shoot forming ability of that tissue. The regenerative potential was reduced with increasing distance from the apical region. In most genotypes, except Enkeim, however, this effect was not dramatic.

4. Morphogenic Studies on the Regeneration Potential of Hypocotyls from Nine Genotypes of A. thaliana

Having designed a culture system for the induction of high frequency regeneration in A. thaliana (see Sections 1 and 2) it was decided to utilise this system to investigate in greater detail the process of organogenesis, especially de novo shoot formation.

By manipulating the duration of the first and second stages while keeping all the other culture parameters constant, and utilising a wide range of genetic sources, it was hoped to obtain greater information regarding, rates of callus formation, the acquisition of organogenic competence and the stability of that property with time in culture, the synchrony of the response and the effect of genotype on these variables. Further, such information would provide data to facilitate an effective anatomical examination of the organogenic processes.

Hypocotyls were chosen as the explant source as they could be produced uniformly and rapidly in large amounts, and more importantly, because they were the most caulogenic tissue source (see Sections 2 and 3). These were excised and placed on the first stage medium (10^{-6}M 2,4-D/10^{-7}M BAP) and every second day after explanting, up to a maximum of twenty days, thirty replicates from each genotype were transferred onto the second stage (10^{-6}M BAP). The cultures were examined every two days after subculture and scored for callus growth and root and shoot formation. The experiment was executed twice and the combined results are shown in Figures 9 and 10.

4.1 Caulogenic Response to the Two-Stage Culture System Utilising 2,4-D and BAP

4.1.1 Callus Development during the First Stage

Correlated with increasing time in Stage 1 was the growth and development of the callus formed by the hypocotyl explants.

All genotypes developed to a similar pattern. After explanting the pro-vascular strand was seen to increase in girth along its entire length and especially at both cut ends, where development proceeded most rapidly. After about six days a characteristic bilobed, dumbell shape was apparent to the naked eye. This consisted of a small rounded callus at each end, joined by a narrower region of swollen, but not callused tissue, the latter being formed by lateral growth of the hypocotyl vascular strand (see Plates 2A and 2B). The callus ends increased in size with continuing time on the first stage medium until about fourteen days, after which the smooth inter-callus tissue was reduced by growth of the callus ends towards each other and by callusing of its own tissue. After this time the specimen lost the distinctive dumbell shape to appear increasingly as one large, single piece of callus tissue.

Although all nine genotypes developed along the pattern described above, the rate of growth and eventual size after twenty one days, varied with the tissue's genetic background. Figure 8 shows the growth rates for the nine genotypes in the first-stage medium.

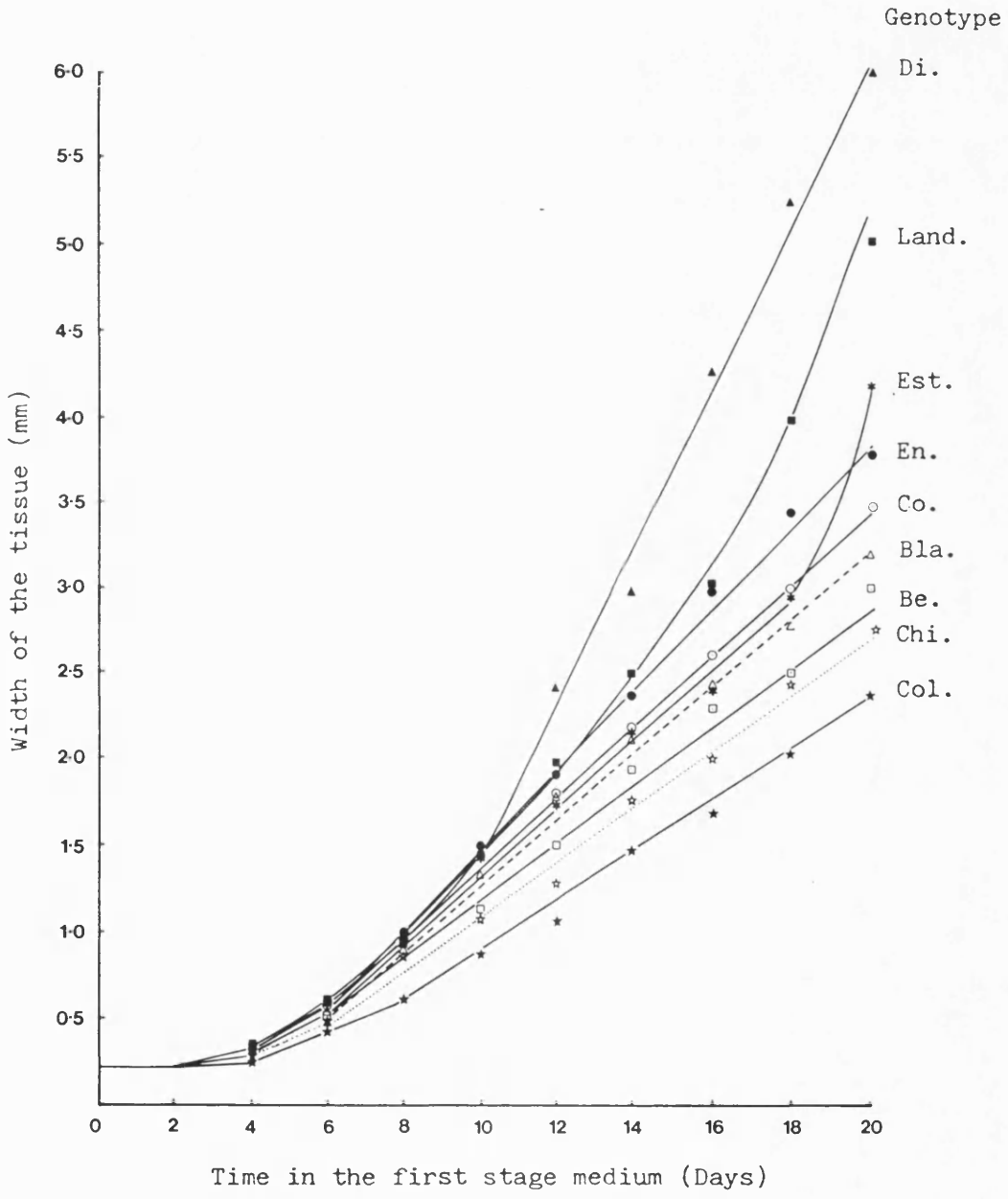
Figure 8 Growth of Hypocotyl Derived Callus from Nine Genotypes of A. thaliana with Continuing Time in the Callus Induction Medium

Hypocotyls were cultured on the first-stage medium used for inducing caulogenic competence for twenty days. The tissue was measured with a graticule every second day across the widest part of the tissue. For the sake of clarity the standard errors are listed in Appendix 3.

Key:

Basal medium	MS supplemented with 2% sucrose
Replicates	30
Growth regulators	10^{-6}M 2,4-D/10^{-7}M BAP

Figure 8 Growth of Hypocotyl Derived Callus from Nine Genotypes of A. thaliana with Continuing Time in the Callus Induction Medium

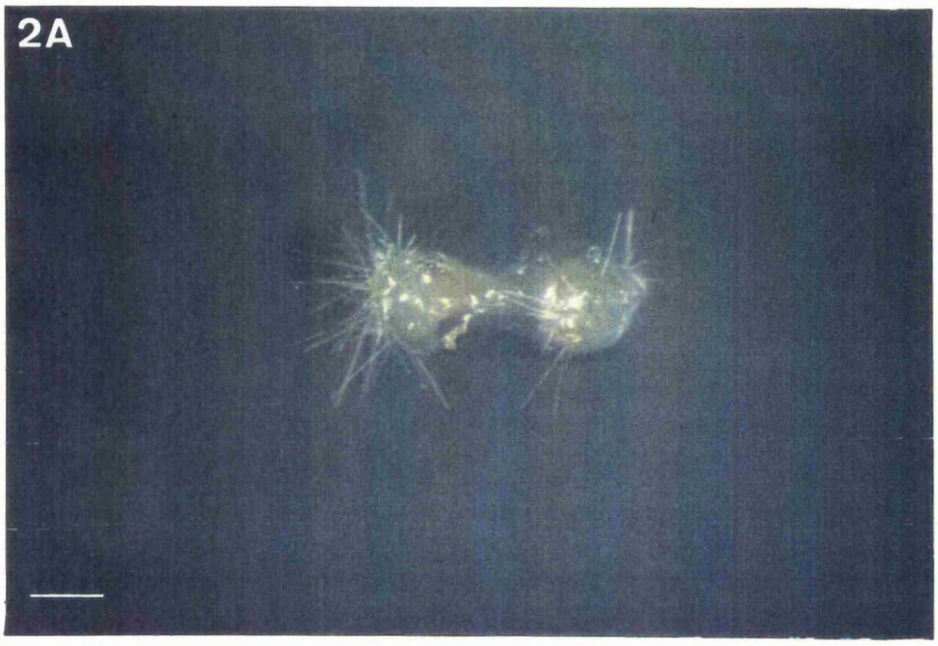


Plates 2A to 2C Formation and Growth of Callus from Hypocotyls of the Genotypes Enkeim and Coimbra

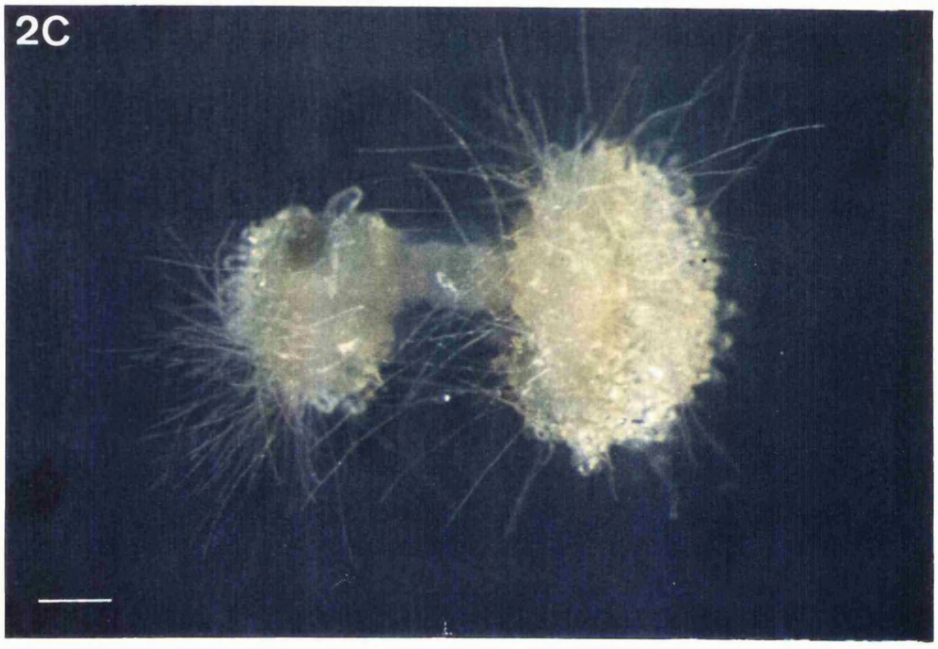
Plate 2A A small dumbell-shaped callus formed from a hypocotyl of Enkeim six days after explanting.
Scale bar = 1mm

Plate 2B Development of the same specimen after ten days on the first-stage medium, showing the increase in size of the calluses at each end and the non-callused nature of the narrower region that connects them.
Scale bar = 1mm

Plate 2C A specimen from the genotype Coimbra 12 days after explanting. Note the distinct dumbell-shape, the non-callused nature of the central region and the "sugary" or "frosty" appearance of the surface of the callus ends. This is a characteristic of this genotype.
Scale bar = 1mm


2A


2B


2C

The size of the callus was assessed by measuring the diameter of the larger (if there was one) callused end at its widest point with a graticule, under the stereo microscope. After an initial lag phase the increase in diameter was constant with time, although for the genotypes Dijon, Landsberg and Estland the growth rate did increase in the latter part of the culture period. The variation in callus growth rates between the genetic types was also constant, and was least striking during the period of maximum competence for shoot formation (6-8 days after explanting, see Section 4.1.2). It is therefore not possible to correlate growth differences between the genotypes with those of caulogenic potential. For example, Enkeim and Coimbra had very similar growth rates at all ages, but vastly contrasting abilities for de novo shoot formation. Likewise Chisdra and Columbia grew at almost equal, and relatively low rates, yet the former is capable of high frequency caulogenesis over an extended first-stage time period, while in the latter competence is short lived.

In addition to differing growth rates, the nine genotypes formed callus with different morphologies. All produced compact tissue in the dumbell shape described above, but four main surface features were also observed which varied between the genotypes. Table 2 lists these differences.

As with growth rates, the presence or absence of hair and the degree of colour or wetness does not equate with shoot regenerative ability. Only the feature of "frostiness" (that is the presence of what appears to be very large vacuolated cells on the surface of the callus, which impart a sugared, or frosty,

Table 2 Morphological Characteristics of the Callus formed by Nine Genotypes of A. thaliana after 21 Days Culture on Media Supplemented with 10^{-6}M 2,4-D/10^{-7}M BAP

	Callus Characteristics				
Genotype	Relative Callus Size	Colour	Hairiness	Wetness	Frostiness
Enkeim	***	XX	+	-	-
Bensheim	**	Wh.	-	+++	-
Dijon	*****	XX	+	-	-
Landsberg	*****	XXX	+	-	-
Estland	***	XXX	+++	-	-
Chisdra	**	XX	+	-	-
Blanes	***	XX	+	-	-
Columbia	**	X	++	+	+
Coimbra	***	X	+	-	+++

Hypocotyls were excised and placed on the first-stage medium. After 21 days culture the calluses were scored for their size, colour and the relative degree of the three major morphological characteristics shown.

Key:

Basal medium MS supplemented with 2% sucrose

First stage medium 10^{-6}M 2,4-D/10^{-7}M BAP

colour: XXX = green

XX = pale green

X = very pale green

Wh. = white

appearance to the tissue) can be correlated with shoot formation. "Frosty" callus was seen to a large degree on Coimbra (see Plate 2C), a considerable extent on Columbia, and rarely, or never, on the other genotypes. Further, it developed on Coimbra callus after about eight days, and was common on Columbia from twelve days onwards. This callus type is thus associated with the low frequency genotypes only, and especially so when they were least competent for shoot induction.

The morphology and anatomy of the regeneration process will be examined in detail in Chapter 2.

4.1.2 Effect of the First Stage Duration on Caulogenesis

The duration of the first stage was the most critical factor governing shoot formation. Transfer after six to twelve days in Stage 1 induced optimum caulogenesis in all genotypes, and in all cases, except Columbia and Coimbra, very high frequencies were obtained; between 95 and 100% of the calluses producing at least one shoot (see Plate 4). Figure 9 shows that a first stage longer or shorter than this considerably reduced the callus's ability to respond to the shoot inducing second stage.

Culture for less than six days in the first stage, failed to induce any shoot formation while a first stage of four days followed by transfer to the shoot induction medium, caused the production, not of shoots, but of a large number of roots (see Figure 12). Rhizogenesis in the second stage is examined in greater detail in Section 4.2.

Figures 9.1 - 9.2 The Effect of the Duration of the First Stage on the Caulogenic Frequency of Hypocotyls from Nine Genotypes of A. thaliana

Hypocotyls were subjected to the two-stage system devised for maximum shoot regeneration in Columbia (see Section 2). Every two days, up to a maximum or 20 after explanting 30 specimens were subcultured onto the second stage. The frequency of caulogenesis was scored 7 and 21 days after subculture. The data from the nine genotypes are presented in two graphs in the interests of clarity.

Key:

Basal medium	MS supplemented with 2%
Replicates	60
First stage	10^{-6}M 2,4-D/10^{-7}M BAP
Second stage	10^{-6}M BAP

Genotypes;	symbol	Genotype
	—★—	Columbia
	—●—	Enkeim
	···☆···	Chisdra
	—○—	Coimbra
	—✱—	Estland
	—▲—	Dijon
	—□—	Benshiem
	--△-·	Blanes
	---■--	Landsberg

Figures 9.1A and B Frequency of Caulogenesis after Various Times on the First Stage and Seven Days on the Second Stage

Figure 9.1A Response of Co., Col., En. and Chi.

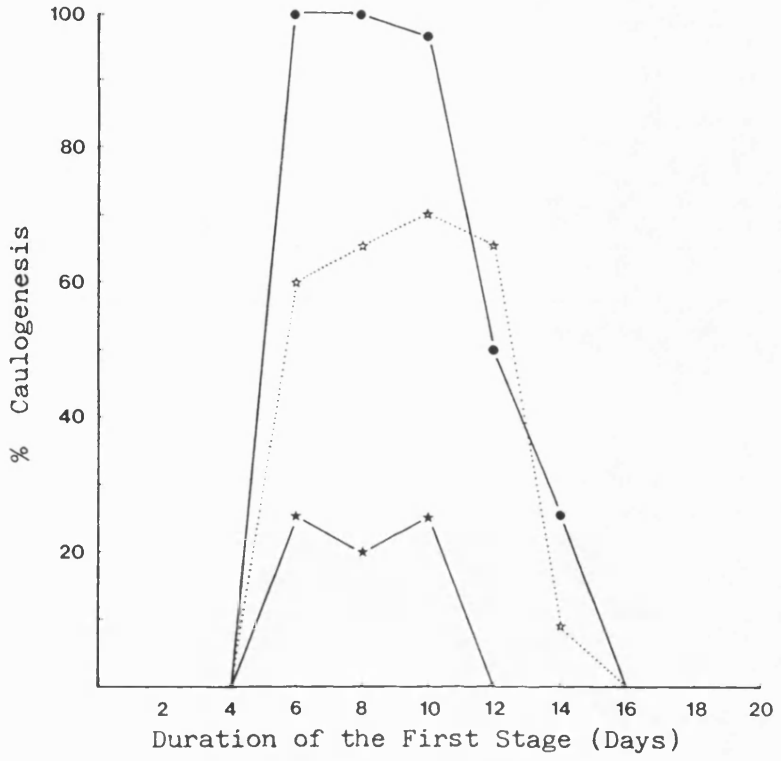
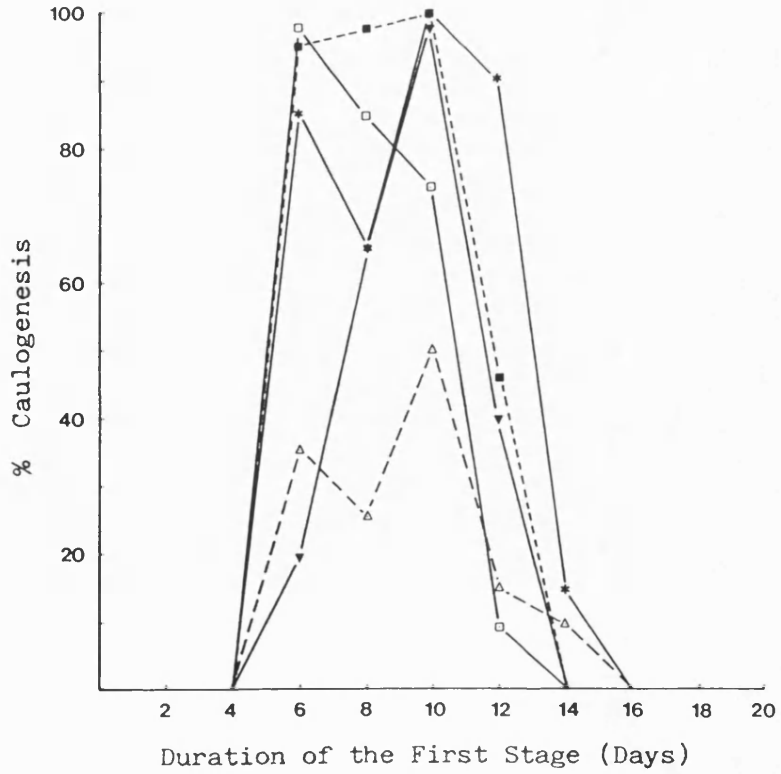


Figure 9.1B Response of Di., Est., Bla., Be. and Land.



Figures 9.2A and B Frequency of Caulogenesis after Various Times on the First Stage and 21 Days on the Second Stage

Figure 9.2A Response of Co., Col., En. and Chi.

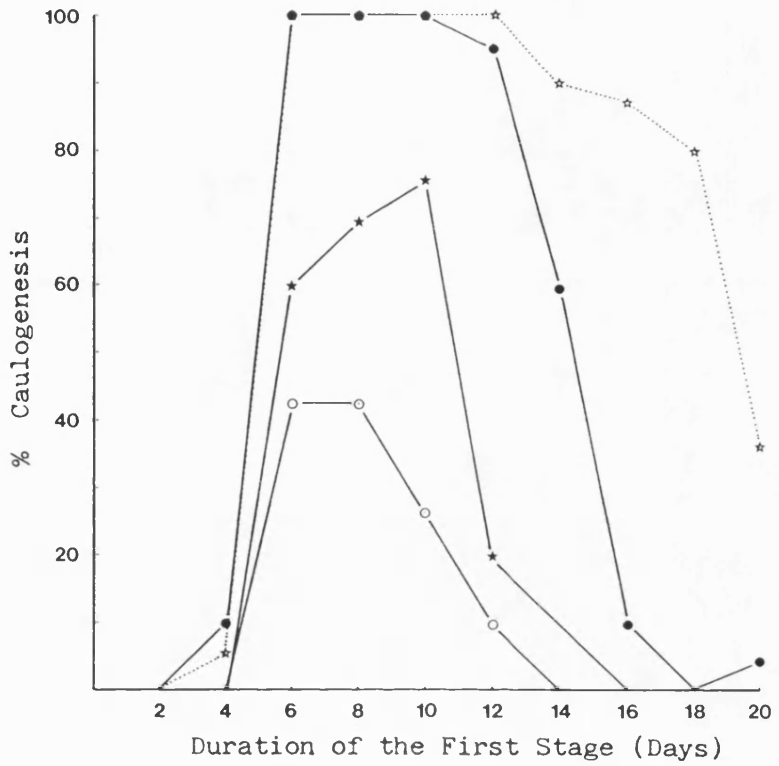
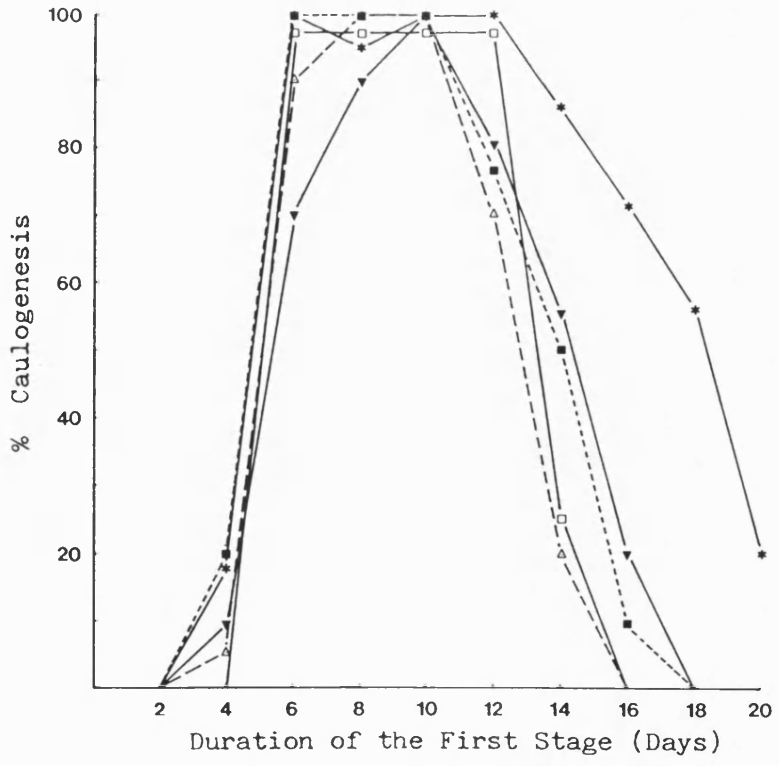


Figure 9.2B Response of Di., Est., Bla., Be. and Land.



Prolonging Stage 1 to exceed twelve days also led to a significant loss in caulogenic competence (see Plate 4B); transfer after sixteen days caused the regeneration frequency to fail from c. 100% to less than 20%, and in some cases to 0%. There were exceptions to this however: Coimbra and Columbia, although behaving in a similar manner, lost their competence for shoot production earlier, at eight and ten days respectively. Conversely, Estland and Chisdra had the ability to form callus which maintained its competence for high frequency caulogenesis beyond twelve days. This is especially true for Chisdra which could still produce regeneration frequencies of around 80% after eighteen days on 10^{-6}M 2,4-D/10^{-7}M BAP (see Plate 4C).

Despite this it is apparant the the duration of the period of maximum competence was clearly defined and relatively short. Increasing time in the first stage was closely correlated with a rapid loss of caulogenesis in the second stage, while a minimum exposure (at least six days) to the first stage was required for that competence to be acquired. The exact timing of the "competence window" varied with the genotype tested.

4.1.3 Effect of the Duration of the Second Stage on Caulogenesis

Figure 10 was constructed from the same data as Figure 9 but it is displayed to show more clearly the effect of continuing second stage culture with respect to the first stage duration and genotype. As in Figure 9 the loss of caulogenic competence with increasing time in Stage 1 is still evident, but here other factors

Plates 3A to 3C Organogenesis from Hypocotyl-derived Tissues of Enkeim

Plate 3A Young roots forming from the middle of the specimen after culture for four days on the first stage medium (MS plus 10^{-6}M, 2,4-D/10^{-7}M BAP) and two days on MS supplemented with 10^{-6}M BAP.
Scale bar = 1mm

Plate 3B Multiple root generation from tissue exposed to four days on the first stage and six days on the second stage medium. All the roots derive from the middle and not the ends of the specimen. Note the lack of any shoot regeneration.
Scale bar = 1mm

Plate 3C Multiple shoot regeneration from hypocotyl derived tissue after six days on the first, and six days on the second stage medium. The shoots arise from the callus tissue at either end of the specimen and not from the central region. Although the latter is hairy and possesses root-like structures no proper roots are produced.
Scale bar = 1mm


3A


3B

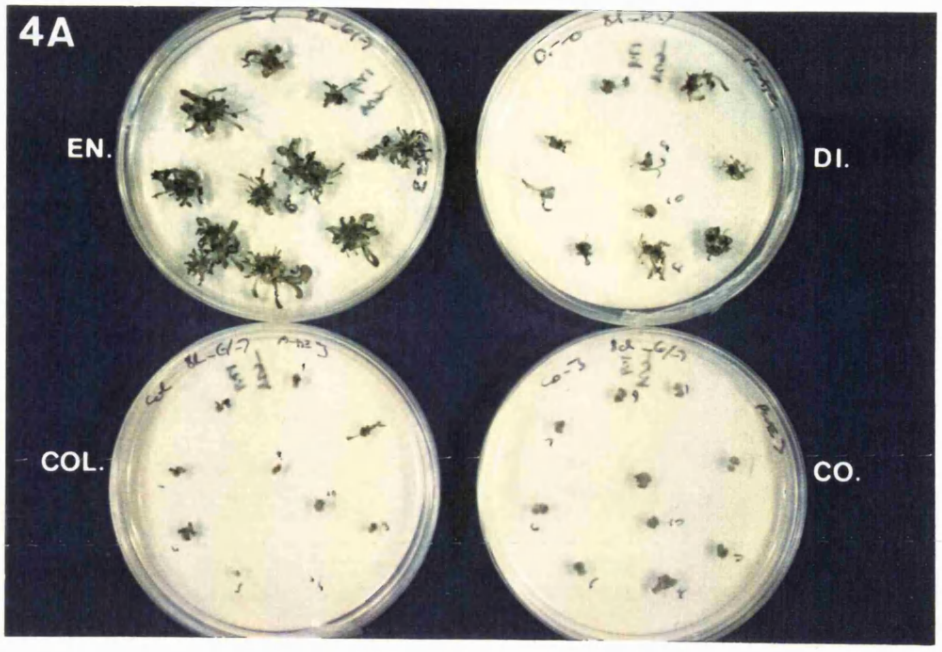

3C

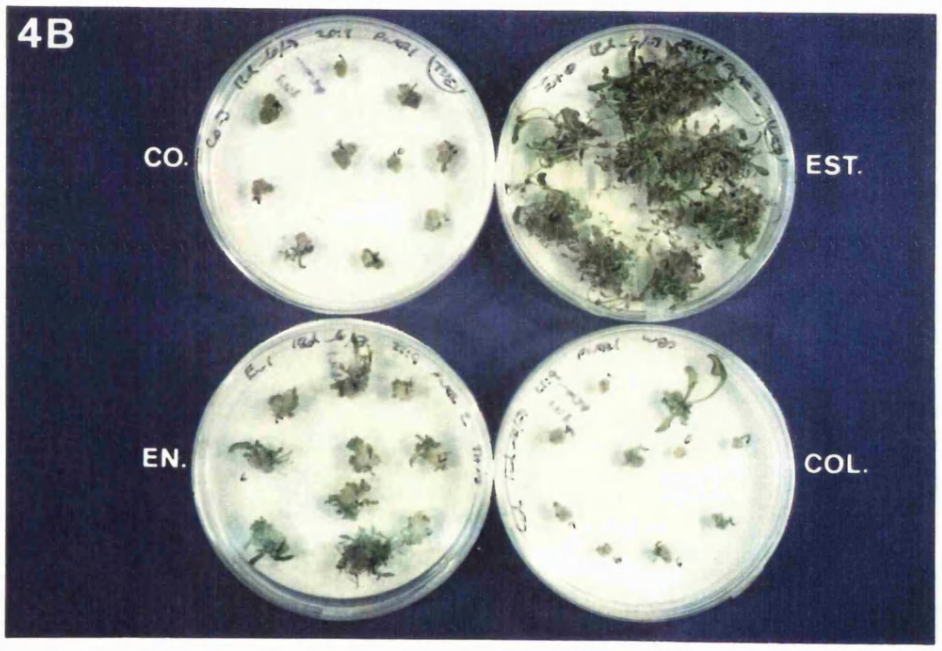
Plates 4A to 4C The Caulogenic Competence of Different Genotypes and the Effect of Increasing Exposure to the Callus Induction Medium

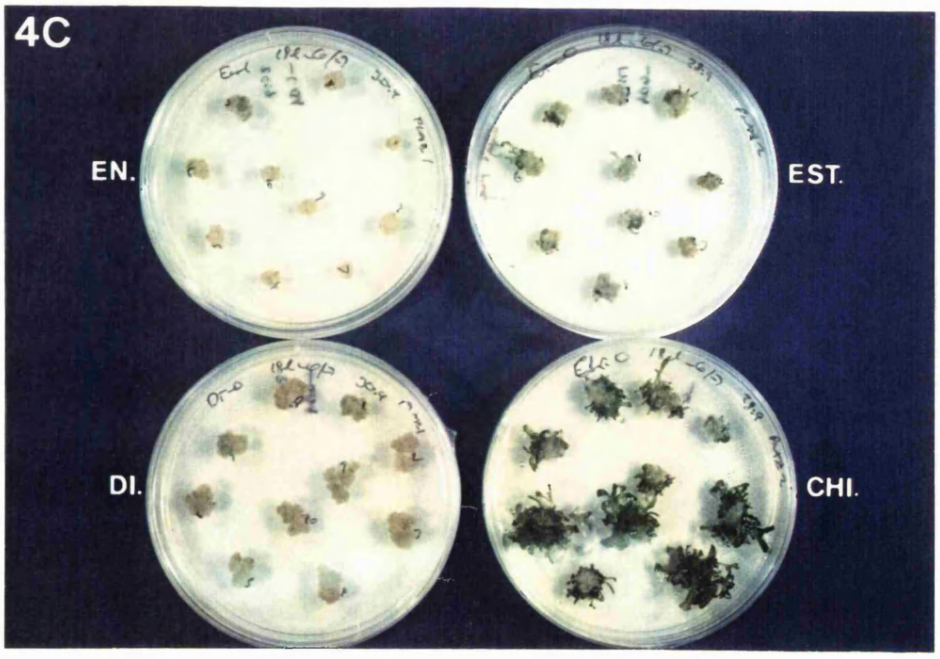
Plate 4A Shoot formation after eight days on the first and 16 days on the second stage. The very high frequency multiple shoot regeneration potential of Enkeim is shown in contrast to the recalcitrant types Coimbra and Columbia. In Dijon the frequency is good but the number of shoots per reaction is low.

Plate 4B Shoot formation is shown after 12 days in the first and 20 days in the second stage. Callus formation is greater than that seen in Plate 4A and the number of shoots produced per reacting callus lower in Enkeim. Coimbra and Columbia are both very poor again but Estland reacts well under this first stage duration.

Plate 4C Shoot formation after 18 days in the first stage and 20 days in the second stage. Large calluses are visible but only Chisdra is capable of caulogenesis. It has a 100% frequency here with numerous shoots formed on each callus.


4A
EN.
DI.
COL.
CO.


4B
CO.
EST.
EN.
COL.


4C
EN.
EST.
DI.
CHI.

Figures 10.1 - 10.9 The Caulogenic Potential of Hypocotyls from Nine Genotypes of A. thaliana to Variation in the Duration of the First and Second Stages Cultures

Figures 10 present the same data as Figures 9 but are displayed to show, in detail, the effect of continued exposure to the second stage medium on tissue cultured for differing times on the first stage. In order to achieve this the plates were scored for shoot formation every two days after subculture.

Key: as Figure 9

Duration of first stage culture (days)	symbol
6	—●—
8	—○—
10	---▲---
12	—□—
14	---★---
16	·····■·····
18	—☆—
20	—*—

Figure 10.1 Enkeim

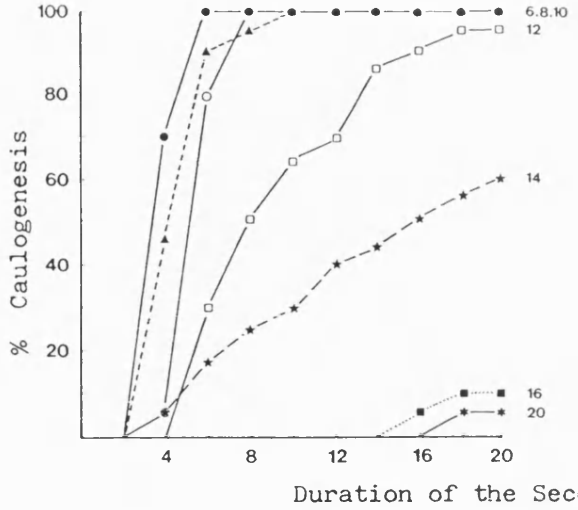


Figure 10.2 Benshiem

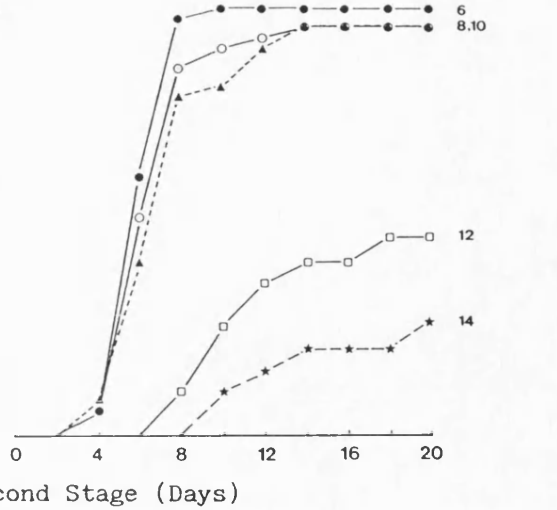


Figure 10.3 Landsberg

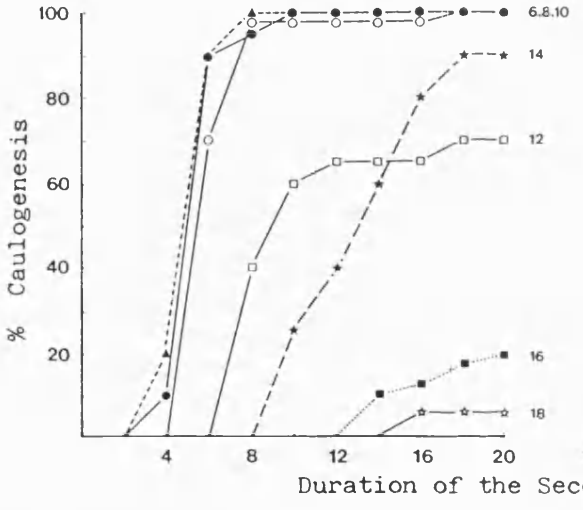


Figure 10.4 Estland

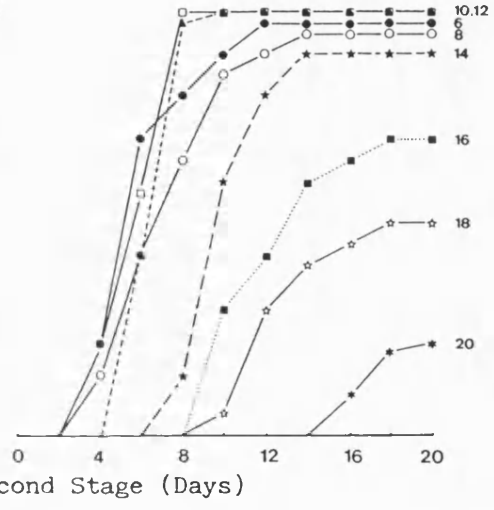


Figure 10.5 Dijon

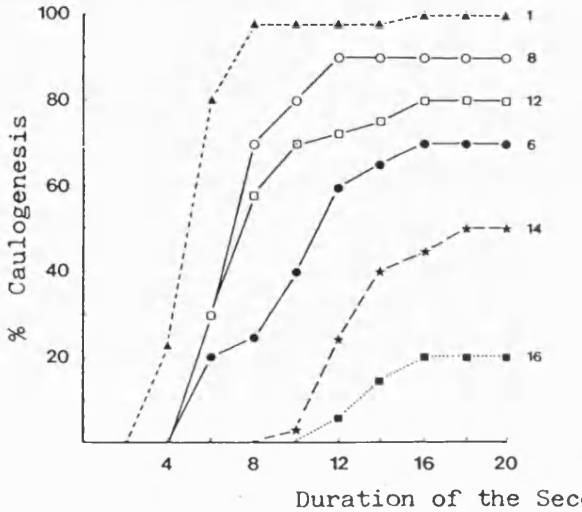


Figure 10.6 Chisdra

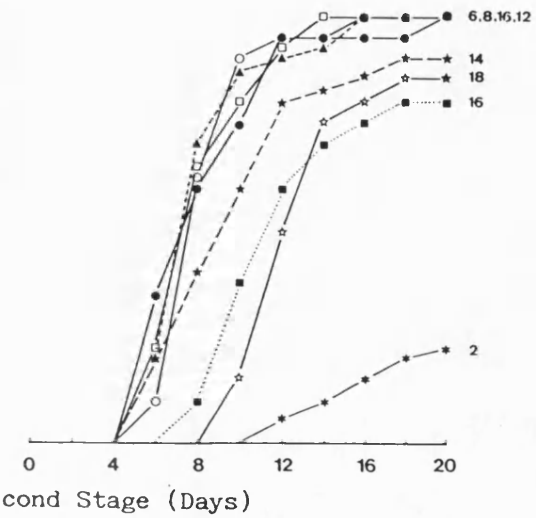


Figure 10.7 Columbia

Figure 10.8 Coimbra

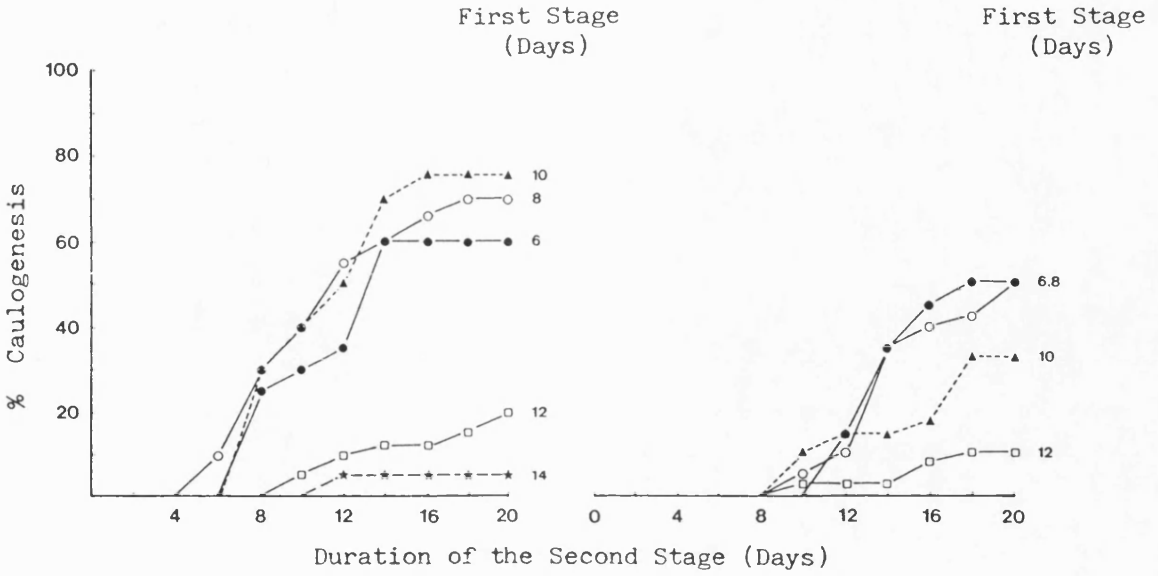
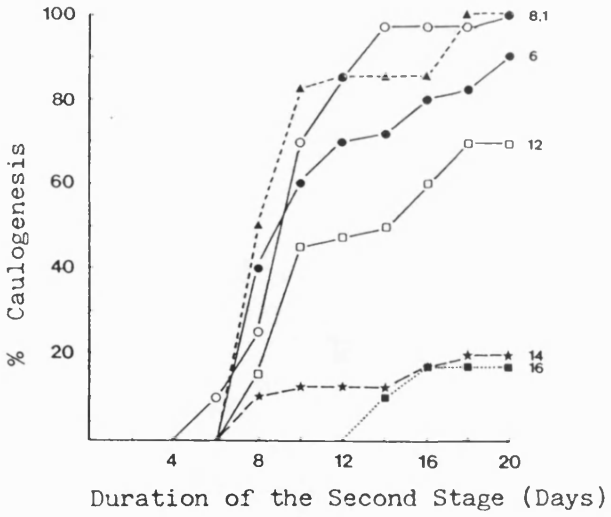


Figure 10.9 Blanes



are also apparant. These are: an increase in percentage shoot formation with continued culture on 10^{-6}M BAP, the presence of a lag period prior to initial shoot emergence and the synchrony of the caulogenic event.

Predictably, the percentage shoot formation increased with continued culture in the second stage up to a maximum. For all first stage durations, however the frequency of shoot formation stopped increasing after a certain time in the second stage medium. The caulogenic processs ceased at this time, and further exposure to 10^{-6}M BAP failed to induce shoot production in those tissues not already responding. For the highly organogenic genotypes under optimum first stages, this is inevitable as no further increase is possible, 100% regeneration having, or almost having, been achieved. In the poorer genotypes and/or after sub-optimal first stages durations shoot production also tailed off, usually after fourteen to sixteen days in stage two.

It would appear that the majority of calluses capable of forming shoots will do so within a short time after subculture, and that although the cultures were scored for only twenty days in Stage 2 one can be confident that no significant shoot production took place after this time.

4.1.4 The Log Phase of the Caulogenic Response

Following subculture there was a lag phase prior to the first shoot buds becoming visible. Shoots were never seen until at least four days after transfer, but the exact timing varied with the duration of the first stage and the genotype.

The longer the first stage the greater was the time before the onset of visible shoot formation. Subculture after six, eight, or ten days on 10^{-6}M 2,4-D/10^{-7}M BAP minimised the lag time, and indeed there is little to separate the times to first shoot formation in these first stages. Delaying transfer beyond this, however, (as well as reducing the total regeneration frequency) considerably increased the lag phase. This was apparent in all genotypes. For example, subculturing Landsberg at ten days or less caused a lag time of about six days, while increasing the first stage to twelve, fourteen, sixteen or eighteen days delayed the onset of shoot production to eight, ten, fourteen and sixteen days respectively.

Although all the genotypes reacted in a similar manner the exact timing of the lag phase varied from one genotype to the next. In Enkeim shoot buds were seen in considerable numbers only four days after subculture, but in Bensheim this takes six, Columbia ten and Coimbra twelve to fourteen days. There is therefore a threefold difference in the lag time between the different genotypes.

4.1.5 Synchrony of the Caulogenic Response

Figure 10 illustrates the percentage shoot formation with time in the second stage medium. As with the total caulogenic response and the length of the lag phase, the synchrony of shoot formation varied with the genotype and the Stage 1 duration. For all genotypes, increasing the first stage duration caused a decrease in the synchrony of shoot formation. This can be clearly

seen when comparing the time from first shoot formation to maximum shoot formation in Enkeim; those with a first stage of six, eight and ten days are considerably shorter than those of twelve and fourteen days.

There are also important genotypic effects on the synchrony of response. Two factors should be considered here. Firstly the speed at which maximum regenerative capacity is reached and secondly the pattern by which the increasing first stage culture reduces this potential: both are controlled by the genotype.

Differences in the rate at which maximum regeneration is achieved is most clearly shown when comparing Landsberg and Columbia. The time between first and maximum response in the former is only four days, while in the latter as much as twelve. The number of new calluses producing shoots per day is much greater in Landsberg.

The degree to which synchrony is lost also varies with the genotype. In Enkeim there is a massive loss between first stages of ten and fourteen days, while in Estland and Chisdra this is less dramatic.

4.1.6 Quantity of Shoots Formed by Each Regenerative Event

The average numbers of shoots produced by each responding callus after various times on the first stage plus 20 days on the second stage medium are shown in Figure 11. It should be noted that the values obtained in the more reactive genotypes are only estimates as obtaining counts was complicated by suspicions of

Figures 11.1 - 11.2 The Quantity of Shoots Produced by Each Responding Callus From Nine Genotypes of A. thaliana Cultured for Various Times in the First and 20 Days in the Second Stage

Hypocotyls were excised and subjected to the same two-stage experimental procedure described in Figures 9 and 10. After 21 days in the second stage the dishes were opened and each responding specimen examined to determine the number of shoots produced.

Key:

Basal medium	MS supplemented with 2% sucrose
Replicates	20 (or less depending on the number of calluses forming shoots)
First stage	10^{-6}M 2,4-D/10^{-7}M BAP
Second stage	10^{-6}M BAP

Figure 11.1 Response of Enkeim

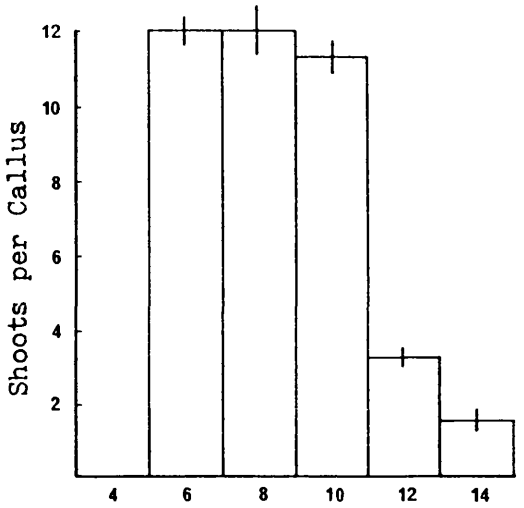
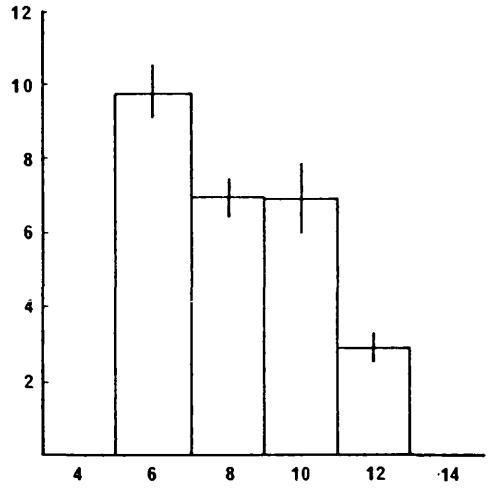


Figure 11.2 Response of Bensheim



Time in the First Stage (Days)

Figure 11.3 Response of Landsberg

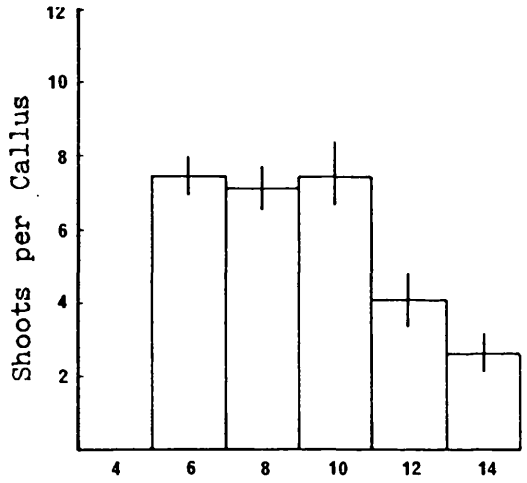
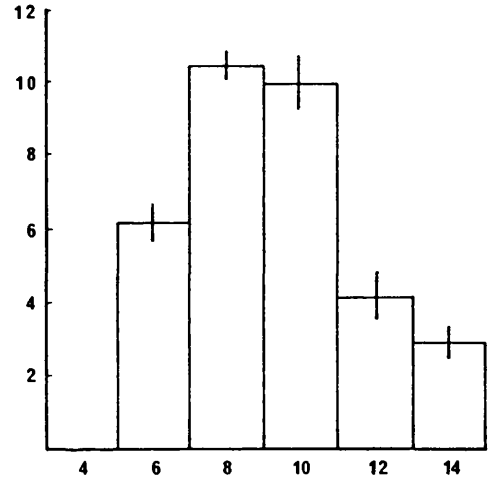
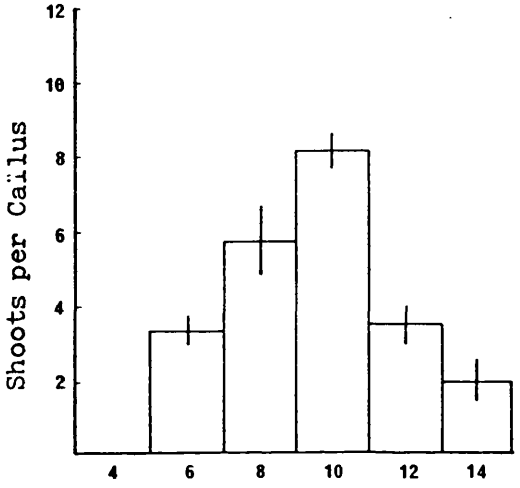


Figure 11.4 Response of Estland



Time in the First Stage (Days)

Figure 11.5 Response of Dijon



Time in the First Stage (Days)

Figure 11.6 Response of Chisdra

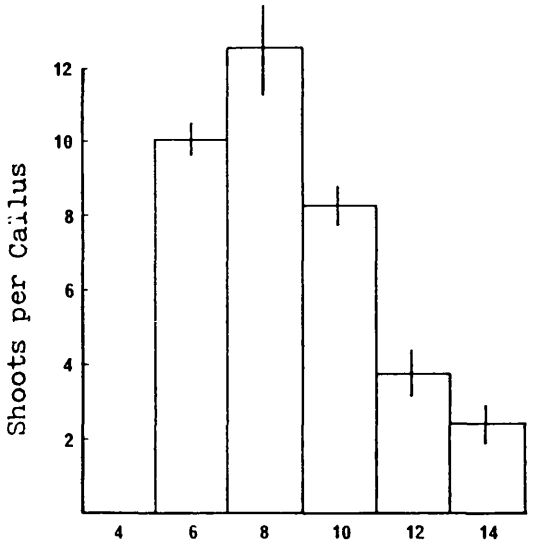
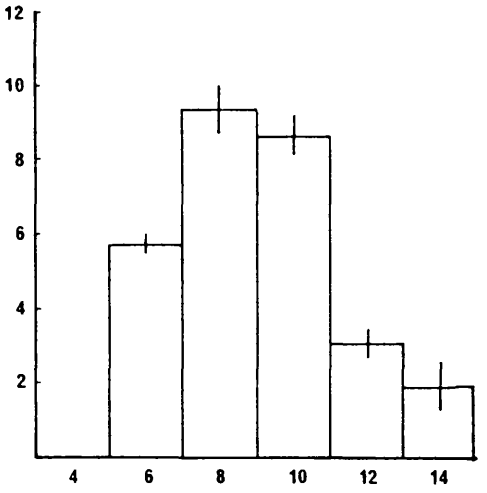


Figure 11.7 Response of Blanes



Time in the First Stage (Days)

Figure 11.8 Response of Coimbra

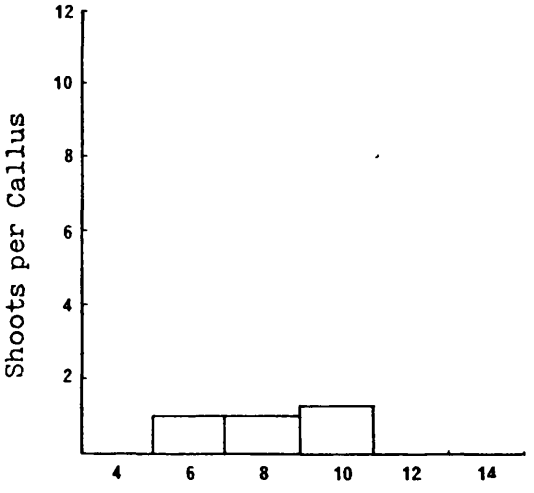
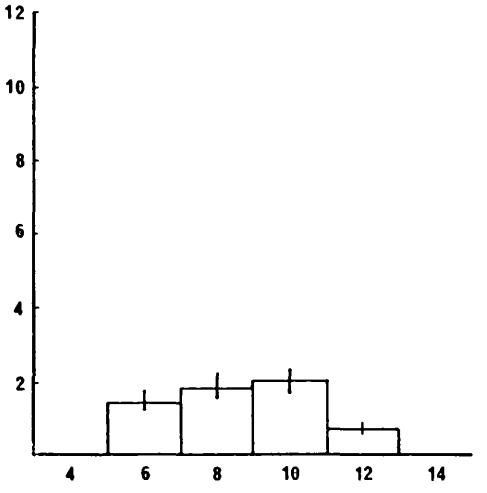


Figure 11.9 Response of Columbia



Time in the First Stage (Days)

secondary caulogenesis from the stems of the newly formed shoots and by axillary bud release. These processes made accurate counting difficult and it is possible that they contributed partially to the very high values recorded in some of the genotypes.

The number of shoots formed by each responding callus varied, like the regeneration frequency, with the duration of the first stage and the genotype.

Common to all nine genotypes was a fall in the number of shoots formed with increasing time spent in the first-stage medium. The critical time appeared to be 10 days, as culture for longer than this resulted in a considerable reduction in the quantity of shoots produced per callus.

The ability to produce multiple shoots from each calluss varied strikingly between the nine genotypes; Enkeim, Estland, Bensheim and Chisdra were all capable of forming between 10 and 12 shoots while Columbia and Coimbra could produce only one, or occasionally two, on each of its caulogenic calluses.

4.1.7 Effect of Genotype on Shoot Regeneration

The response of the nine genotypes subjected to this regeneration system has already been dealt with in each of the previous Sections. These show conclusively that the genotypes differed in all aspects of the shoot forming process: the regeneration frequency, duration of the period of competence in the first stage, length of the lag phase, synchrony of shoot production and the quantity of shoots produced. A genotype tended to be

consistent across these variables; if it scored highly for one it would do so for all. Columbia and Coimbra were clearly the most recalcitrant with respect to de novo shoot formation, as they scored poorly for all the variables examined.

4.2 Rhizogenic Response to the Two-Stage Culture System

Roots were never observed during the first-stage culture period on 10^{-6}M 2,4-D/10^{-7}M BAP, but were produced to varying degrees after transfer to the second stage. The duration of culture on the first stage determined the extent of rhizogenesis from the hypocotyl explants. The percentage root formation with increasing time in the first stage and twenty days in the second stage is shown in Figure 12.

Figure 12 shows that high frequency rhizogenesis took place from two distinct first stage periods: four days and 12-16 days. Subculture prior to four days produced no significant root or shoot formation, thus the tissue has no morphogenetic competence until cultured for a minimum of four days. Transfer at this time, however, induced the largest rhizogenic response recorded. This occurred in all the genotypes with most reaching frequencies of between 90 and 100%. At this stage callus formation was underway, but minimal, and all the roots originated from the central, swollen region, not the callused tissue (see Plates 3A and 3B). The rhizogenetic event took place rapidly, and was highly synchronous; roots becoming visible one to two days after subculture.

Figures 12A and B Rhizogenesis from Hypocotyl-Derived Calluses of Nine Genotypes of A. thaliana After Various Times on the First-Stage and 21 Days on the Second-Stage Medium

Figures 12 present data for root formation gathered from the same experiment as described for shoots in Figure 10. The experimental details are therefore identical.

Two graphs have been produced here in the interests of clarity only as plotting all nine genotypes together was found to be confusing.

Key: Experimental details as in Figure 10

Genotype	Symbol
Enkeim	—●—
Estland	—*—
Bensheim	—□—
Landsberg	---■---
Blanes	- -△- -
Dijon	—▲—
Chisdra	···☆···
Columbia	—★—
Coimbra	—○—

Figure 12A Rhizogenic Response of the Genotypes Enkeim, Estland, Benshiem and Landsberg

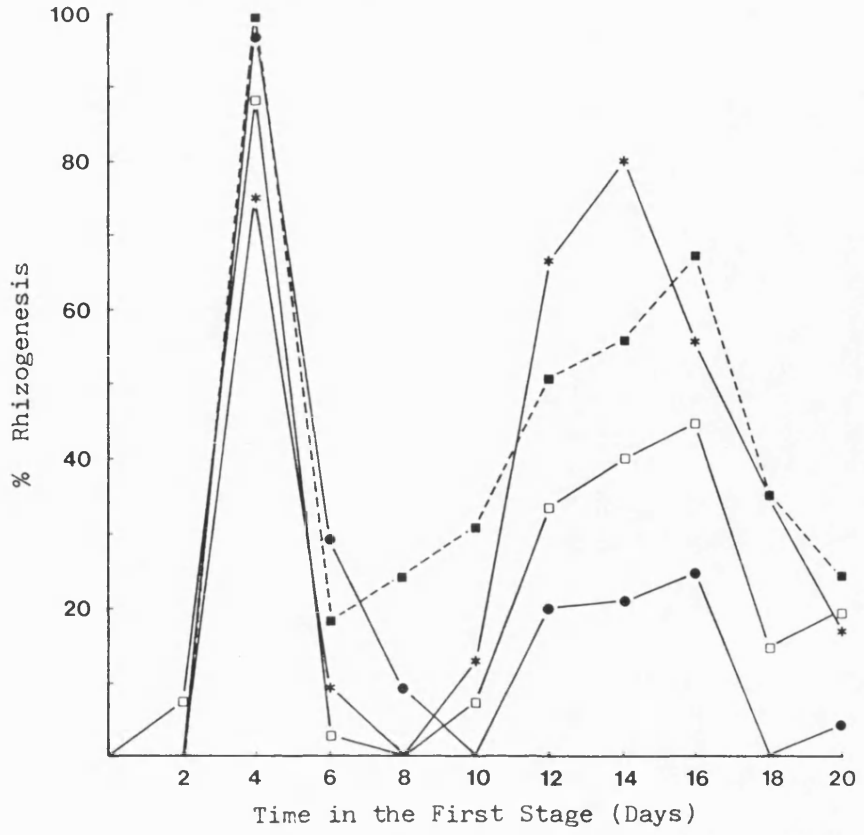
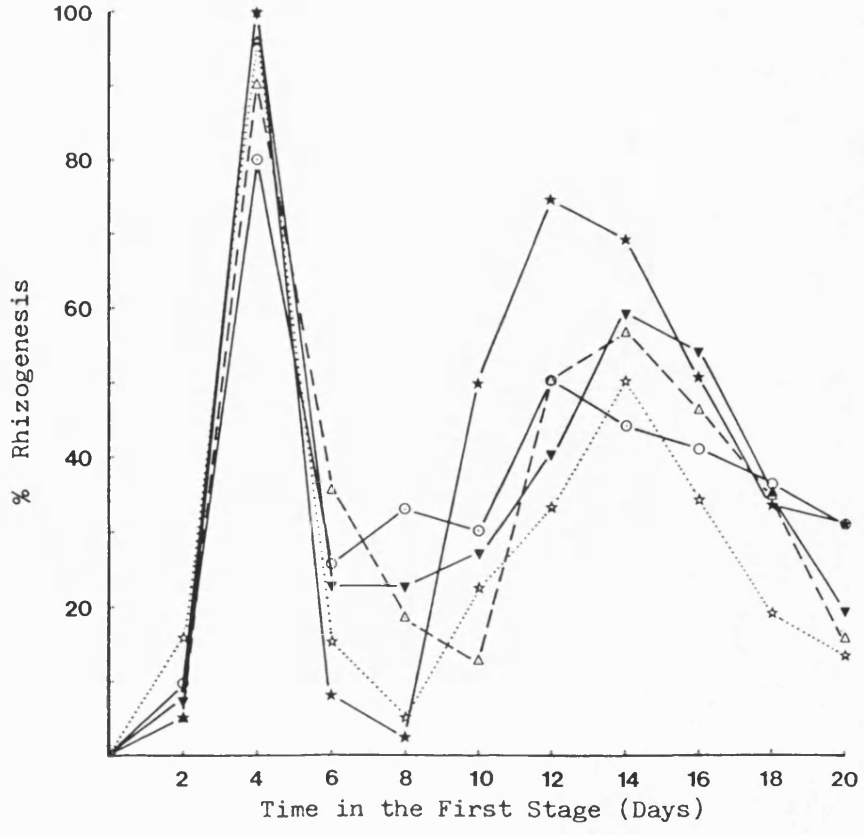


Figure 12B Rhizogenic Response of the Genotypes Blanes, Dijon, Chisdra, Coimbra and Columbia



Delaying transfer until six or eight days caused a dramatic fall in the ability of the calluses to form roots. Once again this occurred across the range of genotypes studied, although the exact values reached did vary. Six genotypes recorded frequencies at 10% or below. This period of reduced rhizogenic competence corresponds with the tissue's time of greatest competence for shoot regeneration.

Prolonging Stage 1 until ten days or longer allowed root formation to be reestablished. All the genotypes responded as such, but this time there was much greater variation in its extent and the phenomenom was sporadic and no longer synchronous. The most notable difference, however, was that these roots arose more from the callus tissue at each end and less from the organised central tissue as in the first rhizogenic event.

It can be seen, therefore that there was two distinct root formation events, and that the characteristics of each were different.

5. Investigation of the Time Needed in the Second Stage to Induce Organogenic Competence

In order to investigate the time needed to induce competence for organogenesis in the second stage, a three stage culture system was devised based on the experiments of Christianson and Warnick (1983;1984;1985). Hypocotyls from the genotype Enkeim were cultured on 10^{-6}M 2,4-D/10^{-7}M BAP for six

days, transferred to 10^{-6}M BAP for differing lengths of time followed by subculture to basal medium without growth regulators. It was hoped that such a three stage system would indicate the time needed in the second stage to induce competence and determination for shoot formation.

Fifty replicates were used for each of the second stage durations tested, and the results are shown in Figure 13.

Increasing the duration of culture on 10^{-6}M BAP in the second stage prior to transfer to hormone free medium increased the frequency of shoot regeneration, while decreasing the incidence of root formation.

After six days in Stage 1 the tissue was determined for root production because transfer to the hormone free medium induced very high frequency rhizogenesis. This determination was highly stable as the potential for high frequency root formation was retained even when the tissue was subjected to three days in the shoot inducing second stage. Only when the duration of culture in 10^{-6}M BAP was greater than three days was the potential for root production reduced. By six days the rhizogenic competence was lost, with none of the calluses forming any roots.

Shoot formation followed an opposite pattern with competence for high frequency caulogenesis not being acquired until the culture period in the second stage was at least 24 hours long. It is clear that the tissue is not determined for caulogenesis after the first stage but is competent for induction to this state by the second stage medium. Further, induction for high frequency

Figure 13.1 - 13.2 Effect of Variable Exposure to the Shoot Induction Medium on Determination for Organogenesis in a Three Stage Culture System in the Genotype Enkeim

Hypocotyls of the genotype Enkeim were exposed to a three stage culture system with varying time on the second stage. After 21 days on the third stage the plates were scored for the frequency of shoot and root formation.

Key:

Basal medium	MS supplemented 2% sucrose
Replicates	50
First stage	6 or 12 days on 10^{-6}M 2,4-D/10^{-7}M BAP
Second stage	varying times on 10^{-6}M BAP
Third stage	21 days on hormone free medium

roots - --O--

shoots - —●—

Figure 13.1 Effect of Variable Exposure to the Second-Stage Medium after 6 Days in the First Stage and 21 Days in the Third Stage

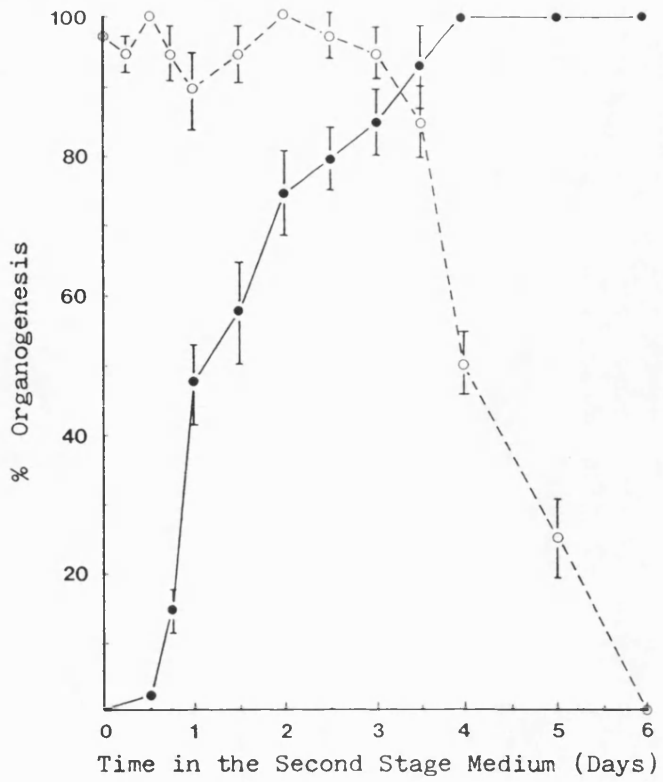
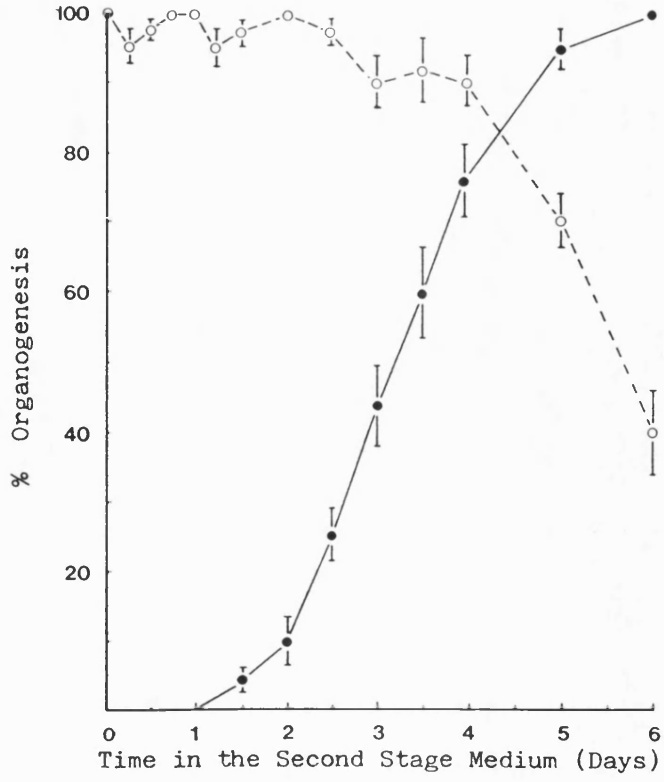


Figure 13.2 Effect of Variable Exposure to the Second-Stage Medium after 12 Days in the First Stage and 21 Days in the Third Stage



shoot regeneration was not achieved until a duration of three days on the BAP medium was reached, and the normally very high caulogenesis associated with Enkeim achieved.

The same pattern of declining rhizogenesis and increasing caulogenesis with increasing exposure to the shoot induction medium was seen when the tissue was given a 12 day treatment on the first-stage medium (see Figure 13.2). In comparison to the shorter first stage, however, the reduction in root formation was delayed until four days instead of three and does not reach zero when exposed to 10^{-6}M BAP for six days. With regard to shoot formation. 48 hours was required on the second stage to induce any shoot regeneration and the frequency of this response increases more slowly than it did for the tissue cultured for six day on the first-stage medium.

As in previous experiments the individual calluses were capable of forming both roots and shoots, depending on the culture regime to which they were exposed. This phenomenon was apparent here, especially when the second-stage exposure was between one and three days. It should be stressed, however, that in the case of the six-day first-stage the two organ types arose from different and highly distinctive, parts of the callus. All the roots were produced by the central, thin, smooth, cylindrical tisssue, which was formed between the callused ends, and never, or very rarely, from the callused ends themselves. The shoots, on the other hand, were always formed from the true callus tissue and never from the organised central region. The situation in the twelve day first stage was less clear as the larger size of this tissue, especially

after time in the second and third stages, and associated loss of the bi-lobed shape, made it difficult to identify the exact origin of the shoots and roots. The tissue types, and the cellular organisation leading to root and shoot formation, are illustrated and described in Chapter 2.

Although the number of shoots formed by each regenerative event was not recorded in this experiment, visual inspection, as seen in Plate 5, revealed that the quantity of regenerants increased, like the regeneration frequency, with increasing time in the second stage medium.

6. Generation of PE Tissue from the Genotype Columbia and Investigation of its Suitability for the Production of Somatic Embryos

6.1 The Production of PE Tissue

In Section 2.2.2.4 the production of a tissue type considered to be potentially embryogenic (PE) was described and illustrated in Plate 1. This was generated when hypocotyls and cotyledons were cultured for about 20 days on basal medium supplemented with 10^{-6}M 2,4-D and 10^{-7}M BAP. The genotype Columbia was assessed to form PE tissue of the best quality and at the greatest frequency (see Section 3.1.4). In order to investigate the production of this tissue further, hypocotyls and cotyledons from Columbia were cultured on various media with differing

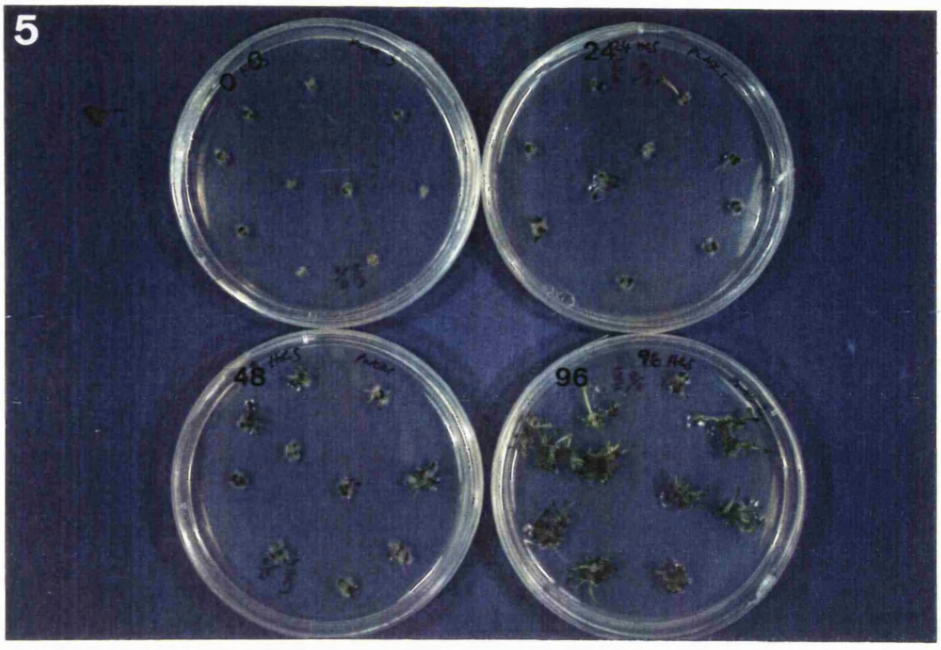
Plate 5 Effect of Increasing Exposure to the Second - Stage Medium on Determination for Shoot Regeneration

After culture on the first-stage medium for six days increasing exposure to the second stage before transfer to basal medium (the third stage) increased the frequency of caulogenesis and the number of shoots produced by each response. This Plate illustrates the effect of the duration of exposure to the second stage on shoot regeneration. A minimum of 24 hours was needed on the second stage to impart determination, and the quantity of the response increases with increasing exposure to the 10^{-6}M BAP medium.

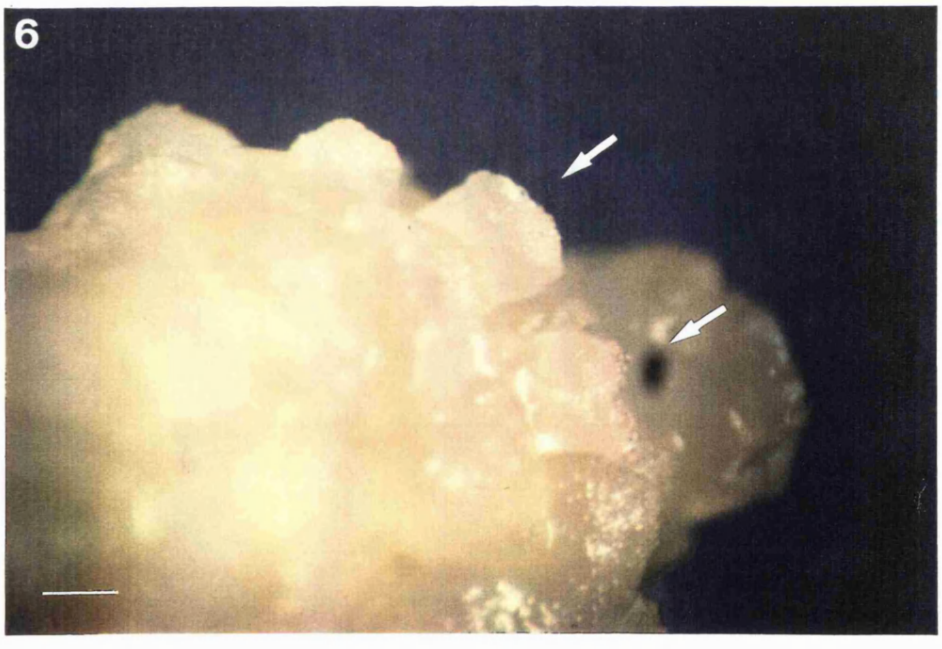
Plate 6 The Appearance of Amomalous Structures on the Surface of Callus Derived from Hypocotyls of Columbia

Embryo-like structures (arrowed) seen on callus derived from hypocotyl tissue from the genotype Columbia 21 days after explanting.
Scale bar = 200μm

5



6



concentrations and types of cytokinins, sugar concentrations and light regimes. Thirty replicates were used for both explant types and for each of these media, and any callus with PE tissue extending over more than one quarter of its surface after 21 days was scored as a positive result. The results are shown in Table 3.

The potential to produce PE varied with the explant type, illumination, sucrose concentration and the growth regulator types and concentrations. Of all the systems tested the best results were achieved from hypocotyls cultured on a basal medium supplemented with 10^{-6}M 2,4-D/10^{-7}M BAP with 20g/l sucrose in the light (16hr daylength).

6.1.1 Effect of Explant Type

Hypocotyls were consistently the more effective tisssue type for formation of PE tissue.

6.1.2 Effect of Illumination

Culture under light conditions was superior to that of continuous dark in both explant types, at both sucrose levels. There were exceptions to this however, especially when zeatin was the cytokinin present; here the light/dark effects were minimal or absent.

6.1.3 Effect of Sucrose Concentration

In most cases the level of sucrose had a strong effect on PE production. In the case of otherwise optimum culture conditions (10^{-6}M 2,4-D/10^{-7}M BAP in the light) doubling the sugar

Table 3 Callus Formation and PE Tissue Production from Hypocotyls of Columbia After 28 Days Exposure to Various Culture Regimes

Hypocotyls and cotyledons were excised and placed on media containing concentrations of 2,4-D in combination with BAP, kinetin or zeatin with one of the two sucrose concentrations in either 16 hrs. light or in continuous darkness. The calluses were scored for their relative size, colour and the percentage that produced PE tissue. An individual was scored positively for the final feature if 75% or more of its surface was covered in this tissue.

Key:

Basal medium		MS supplemented with 2% sucrose
Replicates		50
Callus size		* = minimum to ***** = maximum
Colour:	XXX	= green
	XX	= pale green
	X	= very pale green
	Wh.	= white
	Ye.	= yellow

Culture Variables

(1) Growth regulators

(2) illumination

(3) sucrose concentration (g/l)

Table 3 Degree and Type of Callus Formation from Seedling Explants of Columbia after 21 Days Exposure to Various Culture Conditions

Culture Variables			Explant Type					
			Hypocotyls			Cotyledons		
(1)	(2)	(3)	Callus	Colour	% PE	Callus	Colour	% PE
10^{-6}M 2,4-D + 10^{-7}M BAP	Lt.	20	****	X	86	***	XX	60
	Lt.	80	***	X	44	***	XX	26
	Dk.	20	***	Wh.	68	***	Wh.	28
	Dk.	80	***	Wh.	32	***	X	28
10^{-6} 2,4-D + 10^{-7}M Z	Lt.	20	***	X	48	***	XX	22
	Lt.	80	***	XX	28	***	XX	25
	Dk.	20	***	Wh.	28	***	Wh.	28
	Dk.	80	***	Wh.	54	***	Wh.	8
10^{-6}M 2,4-D + 10^{-7}M K	Lt.	20	***	XX	40	****	XX	28
	Lt.	80	***	XX	16	****	XX	8
	Dk.	20	***	Wh.	24	****	Wh.	24
	Dk.	80	***	Wh.	20	***	Wh.	16
10^{-5}M 2,4-D + 10^{-7}M BAP	Lt.	20	*	X	0	***	XX	0
	Lt.	80	**	Ye.	8	***	XX	0
	Dk.	20	*	Ye.	0	***	Ye.	0
	Dk.	80	*	Ye.	0	**	Ye.	0
10^{-5}M 2,4-D + 10^{-7}M Z	Lt.	20	*	X	0	***	XX	0
	Lt.	80	**	Ye.	0	***	XX	0
	Dk.	20	*	Ye.	0	***	Ye.	0
	Dk.	80	*	Ye.	0	**	Ye.	0
10^{-5}M 2,4-D + 10^{-7}M K	Lt.	20	*	X	0	**	XX	0
	Lt.	80	*	X	0	***	XX	0
	Dk.	20	*	Wh.	0	***	X	0
	Dkr.	80	*	Ye.	0	**	Ye.	0

concentration lead to a halving of the PE percentage. This occured in both the explant types. Only with zeatin in the dark, did increasing the level of sucrose cause an increase in PE formation.

6.1.4 Effect of Growth Regulator Type and Concentration

Both the concentration of 2,4-D and the type of cytokinin employed affected the efficiency of PE production.

Increasing the auxin concentration tenfold had a dramatic effect, with the higher level being totally ineffective, regardless of the other culture parameters.

Of the three cytokinins, BAP was much superior to zeatin and kinetin.

6.2 The PE Tissue as a Source for the Production of Somatic Embryos

Having devised a system to produce optimum amounts and quality of PE tissue, attempts were made to induce this tissue to differentiate somatic embryos. This was approached by generating large amounts of PE tissue via the system described above, followed by subculture onto a variety of second stage media supplemented with a range of growth regulators and nitrogenous compounds. 25 to 30 replicates were used for each of these. The composition of the second stage media and the response of the calluses to them after transfer plus 21 days are shown in Tables 4A and 4B.

Tables 4.1 - 4.2 Response of PE Tissue Generated from Tissue of the Genotype Columbia to 21 Days Culture on Various Second Stage Media

PE tissue was obtained by culturing the explants on the media found to be optimal for its formation (see Table 2). Any calluses with 25%, or more, of their surface covered in PE tissue were subcultured onto one of the second stage media.

Key:

Basal medium	MS supplemented with 2% sucrose
Growth regulators	10^{-6}M 2,4-D/10^{-7}M BAP
Culture Conditions	21 days in culture with 16 hrs. daylength
Replicates	20
Callus size	* = minimum to ***** = maximum
Callus colour	+++ = green
	++ = pale green
	+ = very pale green

Table 4.1 Response of Hypocotyl Derived PE Tissue from the Genotype Columbia to Various Second-Stage Media

	SECOND STAGE MEDIA							RESPONSE				
MEDIUM	BAP	Z	CH(g/l)	GLUT(g/l)	NH_4SO_4	IAA	SUCROSE(g/l)	CALLUS	COLOUR	% ROOT	% E.T.	EMBRYOS
1							20	***	+	55	15	0
2	10^{-7}						20	***	+	30	25	0
3	10^{-6}						20	****	+	0	40	0
4	10^{-6}		0.4				20	****	+	0	95	0
5	10^{-6}			1.0			20	***	+	0	50	0
6	10^{-6}				2.5×10^{-2}		20	****	++	15	35	0
7	10^{-6}			1.0	2.5×10^{-2}		20	****	+	0	25	0
8	10^{-6}					10^{-6}	20	****	+	0	60	0
9	10^{-6}		0.4			10^{-6}	20	****	+	10	65	0
10	10^{-6}		0.4				20	****	+	0	60	0
11	10^{-6}					10^{-6}	20	****	+	0	60	0
12	10^{-6}		0.4				80	***	+	0	55	0
13			1%	CHARCOAL			20	***	+	65	5	0

KEY: GLUT = glutamine
E.T. = embryogenic tissue

Table 4.2 Response of Cotyledonary Derived PE Tissue from the Genotype Columbia to Various Second-Stage Media

	SECOND STAGE MEDIA							RESPONSE				
MEDIUM	BAP	Z	CH(g/l)	GLUT(g/l)	NH_4SO_4	IAA	SUCROSE(g/l)	CALLUS	COLOUR	% ROOT	% E.T.	EMBRYOS
1							20	***	+	30	5	0
2	10^{-7}						20	****	+	15	10	0
3	10^{-6}						20	****	+	0	10	0
4	10^{-6}		0.4				20	****	+	0	5	0
5	10^{-6}			1.0			20	*****	+	0	0	0
6	10^{-6}				2.5×10^{-2}		20	****	+	10	5	0
7	10^{-6}			1.0	2.5×10^{-2}		20	****	++	10	0	0
8	10^{-6}					10^{-6}	20	****	+	10	0	0
9	10^{-6}		0.4			10^{-6}	20	****	+	0	0	0
10	10^{-6}		0.4				20	****	+	0	10	0
11	10^{-6}					10^{-6}	20	*****	+	0	15	0
12	10^{-6}		0.4				80	***	+	10	0	0
13		1% CHARCOAL					20	***	+	40	0	0

KEY: GLUT = glutamine
E.T. = embryogenic tissue

None of the thirteen media was effective at promoting the formation of somatic embryos. Inclusion of a nitrogen source, a different cytokinin, a weak auxin, increased sucrose concentration and activated charcoal all proved equally ineffective at inducing the PE tissue to form any embryo or embryo-like structure.

The only morphogenetic event observed was rhizogenesis, with subculture to the hormone-free and the activated-charcoal media causing about 50% and 70% of the calluses respectively to form roots. Increasing the cyokinin concentration suppressed this frequency.

The best result that could be obtained was to maintain the presence and/or induce further formation of PE tisssue. However, continuing culture in the second stage decreased the percentage of calluses with this tissue type. In all cases, except Media 4, which included a casein hydrolosate supplement, the percentage PE tissue was reduced, and in cotyledons effectively erradicated in Stage 2. The PE tissue lost its smooth, shiny, knobbly appearance to become roughened, wet and often hairy.

Of the two explant types hypocotyls were superior at maintaining their percentage PE tissue.

D I S C U S S I O N

DISCUSSION

In this chapter a simple caulogenic culture system was developed which has greater rapidity and synchrony of reaction, superior frequency of response with larger numbers of recovered shoots per responding explant, than any previously reported procedure. This procedure is further enhanced by its effectiveness across a range of genotypes.

The system was developed as a result of a series of investigations examining the major variables of plant tissue culture, but concentrating mostly on the genotype, explant source and the effects of various growth regulator concentrations and combinations on that tissue. In preliminary experiments (Section 1) using hypocotyls from a genotype of unknown origin it was established that, as reported by Negrutiu et al. (1975) and Huang (1985), auxin and cytokinin were needed in order to induce callus formation. For high frequency shoot regeneration, however, 2,4-D in combination with BAP or zeatin were required. These growth regulators were found to be superior to NAA and kinetin and although BAP and zeatin were equally effective (especially in the two-stage system) the substantially lower cost of the former determined its suitability as the cytokinin for further studies.

These results correlate well with other studies with respect to the auxin source. It was clearly established that 2,4-D is superior to NAA at inducing shoot formation, with the latter tending to promote rhizogenesis over caulogenesis (Negrutiu et al., 1975; Gleddie, 1989; Huang, 1985). There has, however, been less agreement about the most effective cytokinin; Negrutiu et al.

(1978b) found zeatin, to be better than kinetin which was better than BAP, and they, along with Huang and Yeoman (1984), Amos and Scholl (1978) and Feldman and Marks (1986) used it extensively in their first and second stage media. The results obtained here indicated both BAP andd zeatin to be considerably superior to kinetin if shoot formation and not callus proliferation is the objective.

Equally striking in earlier studies was the wide use of an auxin in the second stage medium. This was usually in the form of IAA (Negrutiu et al., 1975; Feldman and Marks, 1986: Huang and Yeoman, 1984; and Amos and Scholl, 1978) or occasionally NAA (Acedo, 1986). It was not at all clear if the inclusion of a weak auxin in the second stage was essential or just beneficial, as the workers most often do not quote comparative data for regeneration frequencies with and without it, although Negrutiu (1976) stated that it was not essential. The results obtained here are in agreement with Gleddie (1989) in that the inclusion of IAA in the second stage does nothing to improve the induction of shoots and its presence may in fact promote unwanted rhizogenesis (Acedo, 1986; Gleddie, 1989).

The use of media with multiple growth regulator additions in each stage is commonly reported (George and Sherington, 1984). Whether such treatments are more effective at inducing the desired morphogenic product than simpler media is too often not explained. The system described here was designed intentionally to be as simple as possible and it employed only two growth regulators, one of which is removed in the second stage. Simple media are more cost effective and promote a clearer understanding of the regulatory processes controlling morphogenesis and their degree of

commonality across and within species. There must, therefore, be a case for attempts to keep the growth regulator and other addenda to a minimum, while the benefits of any extra addenda should be clearly demonstrated with the necessary data.

The availability of material of named genetic background, including the commonly used laboratory types Columbia and Landsberg, led to efforts being switched from the unnamed source to these genotypes as this would allow direct comparison to be made with previous investigations. Using these, and especially Columbia, optimum regeneration systems were developed for both one- and two-stage cultures.

A procedure utilising separate callus and shoot induction stages proved to be superior in both the frequency and synchrony of response to one involving continuous culture on the same medium, while it was striking that the medium promoting maximum regeneration in each case was different; in the continuous system 10^{-7}M 2,4-D/10^{-6}M BAP was optimum but in the two-stage, culture a reversed ratio, namely 10^{-6}M 2,4-D/10^{-7}M BAP, prior to subculture was the most effective and shoots were not seen from tissue cultured continuously on this medium.

In the two-stage system the optimum shoot induction medium was effectively one in which auxin was removed and the cytokinin increased to 10 times the level used in the callus induction phase. While this conforms to the classic descriptions of Skoog and Miller (1957) it is possible that caulogenesis from the one-stage system is a result of an anomalous process. In the latter shoots were not seen until the fourth week and only after good callus formation had taken place. It is possible, therefore, that early in the culture period the 2,4-D and BAP interact to

promote cell proliferation and callus formation. With increasing time, however, the relatively low level of 2,4-D (only 10^{-6}M) would probably be metabolised leaving a relatively high level of BAP to develop which would induce the formation of shoots. The correct ratio of the two growth regulators must be supplied if an inductive balance is to develop in the tissue, and certainly 10^{-7}M/10^{-6}M 2,4-D/BAP was the only ratio at which this occured at high frequency (see Figure 6).

Figure 1 also shows that BAP and zeatin are more effective than kinetin at inducing caulogenesis in the one-stage system and this would be in agreement with idea that these are stronger cytokinin agents than kinetin. Certainly Negruitu et al. (1975; 1978a; 1978b) who used kinetin and 2,4-D extensively in their callusing phases never reported shoot regeneration during the four week first-stages that they employed.

These findings raise the question of the advantages of a two-stage over a continuous system. Although plantlets can be regenerated by the latter in a number of species (Patton and Meinke, 1988) it is not certain whether this regeneration was less efficient than might otherwise have been achieved.

It is well established that in most species the level of auxin must be reduced from the relatively high concentrations needed for callus induction before effective shoot regeneration can occur. Utilising a two-stage culture regime or, alternatively, a single-stage regime with a rapidly metabolished auxin such as IAA, allows the system to be developed especially to meet this end. However, the use of a two-stage procedure allows the tissue to be switched to the shoot-inducing medium at a time when the maximum

number of cells are competent for regeneration. This has the effect of optimising the level of response to the shoot induction phase.

Such control is not possible in a single-stage system with the requirements for morphogenesis being supplied from only one medium, and subsequent events controlled by tissue - generated changes in the endogenous levels of the growth regulators. Such a medium must be a compromise between the differing needs of cell proliferation and controlled organisation and is therefore unlikely to be ideally suited to either. This would account for the lower frequency and synchrony of recovered shoots obtained from the one-, as compared to the two-stage system in this study, and raises the question as to whether the frequency of regeneration in other species which utilise one-stage systems may not be improved upon by adopting separate media for callus formation and organ induction.

The two-stage regeneration system developed here revealed a number of important points concerning regeneration in Arabidopsis, some of which were in agreement with previous reports and others not. Three major factors were seen to determine the caulogenic potential of a given culture and these were the explant source, the genotype and the duration of the callus induction phase.

In comparison with other workers the explants tested here responded well. Hypocotyls, for which the system was originally designed, regenerated at 95-100% frequency in all the genotypes except Columbia and Coimbra. While this surpasses all previous reports except that of Feldman and Marks (1986), the other explants also regenerated at favourable frequencies. As with Negruitu et al. (1975), all the explants examined were capable of caulogenesis

although for them leaves and shoots and anthers were the best sources. Under their system no more than 60% of the leaf explants regenerated a shoot while in this study the frequency varied from about 10 to 50% depending on the genotype. Comparisons with the seed material is more difficult, however, because it is not possible to tell which of the seed tissues contributes to callus formation and shoot regeneration.

In this study the foliose structures (cotyledons and leaves) and the petioles all had lower caulogenic potential than hypocotyls and this was especially true of the cotyledons and leaves which tended towards root instead of shoot formation (see Figure 7). The exact caulogenic potential of the foliose structures varied with the genotype within the range 0 - 50% but was considerable lower than the 100% quoted by Feldman and Marks (1986) for leaves from bolted plants. It should be remembered, however, that their system was developed specifically for this type of explant while that used here was designed for hypocotyls. This demonstrates therefore, and indeed Negruitu states this (1976), that the culture system must be adapted empirically for each explant type if maximum caulogenesis from that tissue is desired.

The occurrence of differing organogenic potential between different explants is widely reported (see Introduction), but the reasons for this are not understood. Different explants have different physiology, anatomy and probably varying amounts of competent, or undifferentiated cells (Williams and Maheswaran, 1986). Thus one explant might metabolise the added growth regulators at a different rate than another therefore requiring a different balance of supplied hormones and/or varying exposure to the callus induction medium, in order to achieve shoot

regeneration. Another may contain more cambial tissue and so possess a larger pool of meristematic cells capable of immediate and rapid division. The critical factor, however, is the number of competent cells, be they predetermined cells or culture induced cells, present at the time of subculture.

Only by maximising the number of these cells and the ratio of them to other types is it possible to maximise the desired morphogenic response. This is what Street (1979) states and it is borne out in this study in the observation that although the cotyledons callused to a greater extent that the hypocotyls (see Figure 3) their potential for shoot regeneration was lower. Thus it would appear that the hypocotyl-derived callus contained more cells competent to respond to the shoot induction medium than that of the cotyledon derived callus. Acedo (1986) and Feldman and Marks (1986) also found no correlation between the degree of callus formation and caulogenesis.

In addition to variation in the caulogenic potential between explant sources, the effects of genotype on the ability to regenerate shoots was striking. The influence of genetic background on morphogenesis within the one species is well documented (see Introduction) and has been described in A. thaliana in all the major investigations to date (Negrutiu et al. 1975; 1978a; 1978b; Scholl and Amos, 1980; Feldman and Marks, 1986; Acedo, 1986). In this case significant genetic influence was recorded when hypocotyls were cultured on the two-stage system designed in Section 2.

Subjected to this regime the nine genotypes were split into two distinct groups; the responsive and the recalcitrant. Seven proved to be highly caulogenic and consistently regenerated

shoots at frequencies of 95-100%, while Columbia and Coimbra responded at levels considerably lower, c. 75 and 50% respectively. This relative recalcitrance was emphasised by their performance across all the parameters of caulogenesis. Thus, as well as scoring much reduced regeneration frequencies, Columbia and Coimbra produced fewer shoots per responding callus, had a lower degree of synchrony and a longer lag phase between subculture and the appearance of the first shoots.

Detailed histological attempts to investigate the reasons behind these genetically controlled differences are dealt with in Chapter 2 but both the rates of callus growth and the macroscopic surface characteristics of the developing tissue were recorded at this stage. As we have seen no correlation was seen between the ability to regenerate shoots and callus size but there was some evidence of the presence of large vacuolated cells on the callus surface being related to recalcitrance. This "frosty" tissue, although occasionally seen on most of the genotypes, was present in large amounts only on Coimbra and Columbia.

Comparing these genotype dependent results with those of other workers reveals some interesting points. While Coimbra was easily the poorest type examined here, Negruitu et al (1978a) found it to be more responsive than Columbia and Chisdra. They showed that Coimbra could attain up to 66% shoot regeneration if NAA was used in the callus induction medium, and that this was superior to 2,4-D, which induced a response of only 27%. This demonstrates that, as for the explant type, manipulation of the medium may considerably influence the competence of different tissues for a desired effect, and that each genotype may need to have the culture system adjusted to suit its individual needs. It is possible,

therefore, that using NAA in this study could have improved the regeneration potential of Coimbra. This, however, was not attempted.

Manipulating the culture variables is one approach to the problem of recalcitrance and some consider that this alone is capable of elevating the performance of the poorest to that of the most responsive (Christianson and Warnick, 1984). The other approach is that some genotypes have some inherent factor(s) which reduce the ability to regenerate in vitro and that this cannot be overcome by simple variations of the culture regime. The performance of Columbia in the present and previous studies may provide some evidence for the latter.

When comparing the shoot regeneration frequencies achieved by this genotype by Acedo (1986), Feldman and Marks (1986), Patton and Meinke (1988) and Gleddie (1989) certain consistencies are apparent. In all the past studies the maximum yield achieved was about 60% and this was always lower than that of the other genotypes subjected to the same culture regime. This corresponds well with that found for Columbia here, in which it regenerated at c.75% frequency with a low number of shoots produced by each responding explant, a rate which was considerably below majority of the other genotypes.

The fact that Columbia has been cultured in numerous media with a range of explants, and responds at roughly the same degree in all could indicate that there is some inherent genetic limitation to the caulogenesis in this genotype. If so then this may prove to be a problem as Columbia is the major genotype used by the molecular biologists and genetic engineers when studying Arabidopsis (Meyerowitz, 1987). Its relatively low regenerative

potential could be a serious limiting factor in the efficiency of recovery of transformed plants. On the other hand if there is a genetic basis to its recalcitrance then its relatively well understood genetic composition may provide a tool for the investigation of the genes or gene complexes controlling morphogenesis in vitro.

The work of Damm and Willmitzer (1988), however, achieved 80-90% regeneration from protoplast-derived colonies of Columbia indicating that it is possible to achieve a high frequency response in this genotype. Although it could be that some selection process took place during the formation of these colonies, so removing non-meristematic cells from the experimental group, this does show that manipulation of the culture variables could still help to improve the regenerative capacity of Columbia.

Both the explant and genotype are variables which are fixed prior to the act of explanting. Once in culture, however, the in vitro conditions affect the caulogenetic potential of the selected tissue. In this study, in addition to the growth regulator regime, the duration of the first stage had a dramatic effect on the ability of the tissue to regenerate organs.

The duration of culture on the first stage medium was found to be critical with respect to caulogenesis. Figure 9 clearly shows that even when the optimum type and concentration of growth regulator, had been selected the degree of shoot regeneration was dependent on the time of subculture.

In this system the optimum first stage was between six and twelve days long. Subculture at this time induced the highest frequency response, the greatest synchrony and produced the most shoots per responding callus. Insignificant shoot formation took

place if the tissue was transferred before this, while competence to respond to the shoot induction medium fell dramatically if the first stage was extended beyond twelve days, with little, or no, shoots formed after 18 or 20 days in the callus induction phase. The genotypes Chisdra and Estland, however, were distinctive in their extended periods of competence for caulogenesis. Both were capable of forming shoots from callus after 16 to 20 days on the first stage, although the frequency of this response was less than half than if subculture took place at eight or ten days.

Possible histological factors behind the limited period of caulogenic competence are examined in Chapter 2 and these studies indicate a possible structural cause. One cannot, however, rule out the role of the endogenous growth regulators. It is possible that there is an accumulation of hormones and/or other factors, especially in tissues exposed to the first stage medium for extended periods, which prevents an inductive balance being achieved after transfer to the second stage and thereby blocking caulogenesis. Obviously some biochemical analysis of the tissues involved would have to be carried out in order to investigate this hypothesis fully, but some data available from this study indicate that such carryover effects might influence the regenerative capacity of a given tissue.

From data gathered relating to the effect of continuing culture on the second stage (Figure 10) it can be seen that tissues exposed to the callus induction medium for extended periods, as well as having a reduced caulogenic frequency also have decreased synchrony of response and an increased lag time. Both of the latter could be due to an accumulation of auxin in the tisssue which is carried over in the tissue at subculture. Such older

calluses are much larger than those with short callus induction phases (see Figure 8) and so could carry over more endogenous growth regulators. Once in the second stage these calluses may take longer to achieve the balance of endogenous factors that are inductive to shoot regeneration; thus the longer lag phase, and as this balance will be achieved gradually, the reduced synchrony of response.

A final factor, and one which is more difficult to explain, is the fact that the frequency of shoot regeneration does not increase after 16 days in the second stage (see Figure 10) and therefore all the calluses that are going to respond will have done so by this time. Feldman and Marks (1986) quote a similar effect with, in their case, no new shoots being formed after 14 days in the second stage medium. The reasons for this are not known but the fact that it occurs in both of these systems, with their different explants and media formulations, is perhaps significant.

The defined and limited period of induction for caulogenic competence described above has significance with respect to the "short preculture concept" advocated by Feldman and Marks (1986) and contrasts greatly with the approach adopted by Negrutiu et al. (1975; 1978a; 1978b).

Feldman and Marks (1986) found that a preculture of seven days on their callus induction medium was optimal for shoot regeneration and that the latter fell away dramatically if the first stage was extended. In contrast to this, the work of Negrutiu et al (1975; 1978) and later Huang and Yeoman (1984), showed that morphogenesis was only achieved from long term callus cultures with the most rapid recovery of shoots being after about two months in culture.

Negrutiu does not explain his reasons for concentrating exclusively on long-term callus cultures but this may be due to a continuation of the approach of most workers of the 1960's, and before, where the primary objective was to induce a healthy, viable callus capable of sustained growth. He seems to have extended this approach in an attempt to regenerate from such tissues. The result is that the culture systems of Negrutiu and Huang are very different from those employed here or advocated by Feldman and Marks.

In the long-term systems the aim appears to be to induce a callus which grows well and then produces nodules of green tissue amongst the other callus material. These nodules are the "regeneration centres" (Negrutiu, 1975; Huang and Yeoman, 1984) and the composition of the medium is manipulated in order to maximise their formation and proliferation. The concept behind the short preculture approach is very different, with both the growth regulators and the duration of the first stage being varied, with the latter relatively short. This variable is not investigated by Negrutiu but is considered to be critical, and indeed has been shown to be so, by both this work and that of Feldman and Marks (1986).

The result of fixing the callus induction stage at four weeks may well have been unfortunate for both Negrutiu and Huang, and it might have determined that they would not achieve as great a degree of caulogenesis as might otherwise have been the case. We have seen competence for shoot formation to be restricted to a period early in the culture and that by 20 days this has almost disappeared. Secondly the size of the callus by four or more weeks and its long exposure to the growth regulators,

especially 2,4-D, will cause considerable endogenous effects and cause physiological and structural changes. This will effect the ability of the shoot induction medium to cause the establishment of conditions in the callus that are conducive to shoot regeneration.

With hindsight the wisdom of employing such long term callus cultures for regenerating from Arabidopsis must be questioned. Extended periods in culture may be beneficial if one wishes to encourage the recovery of somaclones and a problem if one is dealing with scarce material that as a result cannot be multiplied without the loss of the desired genetic constitution. However, when dealing with A. thaliana the second of these criteria does not exist and as a major aim would be to recover genetically transformed tissue and to avoid somaclonal variation, rapid, minimal callus regeneration systems are desired.

It would seem therefore that the rapid, short-preculture systems developed over the last few years are a considerable advance over those of a decade or so ago.

Although the two-stage culture system utilising 2,4-D and BAP was designed to induce high-frequency shoot regeneration, roots were also commonly formed, often at high frequency, after subculture to the shoot induction medium. This was somewhat surprising as this medium contained BAP at a level (10^{-6}M) which one would expect to discourage the formation of this organ. The sporadic and unpredictable nature of rhizogenesis in many species has been commented on by Narayanaswamy (1977), and Negruitu et al. (1978a) has reported root regeneration to be commonplace from numerous culture regimes in A. thaliana.

In this study, however, the pattern of rhizogenesis was quite predictable and distinctive. Like shoot formation the degree of root regeneration depended on the duration of the first stage, but was less influenced by the genotype. All nine genotypes produced the two-phase response shown in Figure 12 in which subculture after four days on the first stage medium induced a rapid, extremely high frequency (90-100%) response. This was followed by a rapid drop in potential if the callusing phase was extended to between six and twelve days, after which there was a gradual recovery. The formation of roots at this latter time was, however, much less synchronous, and occurred at a lower frequency, than the initial response.

It is notable that the loss of rhizogenic potential corresponded almost exactly with the time of maximum competence for caulogenesis. Root regeneration took place both before and after the peak of shoot regeneration but not during this period implying that there may be some sort of competition between the two organogenic states. If this is the case then it remains to be seen if this is a passive effect, with the nutrients and growth regulators directed towards caulogenesis at the expense of rhizogenesis, or whether the regenerating shoots actively inhibit root formation in some way.

It was notable that the roots and shoots were produced from different parts of the explant. At day four all rhizogenesis took place from the smooth, non-callused tissue in the middle of the specimen; in the later response the exact origin of the roots was more difficult to determine due to the break up of the central region and the increase in the size of the callus ends from day 14

onwards. It appeared, however, that the majority of these roots arose from the callus tissue produced at each end of the original explant (see Plate) in these older specimens.

Further information concerning the production of shoots versus roots is provided in Section 2. The experiment was designed to replicate those of Christianson and Warnick (1983, 1984, 1985) in an attempt to examine further the processes of competence and determination in A. thaliana. Unfortunately, the terms competence and determination have been used rather loosely in the plant cell and tissue culture literature with very little appreciation of the underlying mechanisms. The work of Christianson and Warnick specifically addresses the problem of competence and determination but again leaves many questions unanswered about these developmental states. It was considered that the regeneration system developed in the present work with A. thaliana might be suitable for investigating some of these questions.

This was approached by exposing the tissue to a three-stage culture procedure (as in Christianson and Warnick, 1984) in which the duration of the callus induction phase was fixed at six and twelve days and that of the second phase varied prior to subculture to a hormone-free basal medium.

If specimens were transferred directly from the callus-induction medium to the basal medium, roots were produced at very high frequencies, but no caulogenesis took place. Following the interpretation used by Christianson and Warnick (1983) such a result would indicate that the tissue was determined for rhizogenesis at the time of subculture, and that this determination was very strong in that exposure to 10^{-6}M BAP for up to three days did not effectively reduce this response; determination for shoot

regeneration, however, did not occur until the tissue had been exposed to the shoot-induction medium for a minimum period of 24 hours.

Interpretation of these results are complicated to some degree by the effects of growth regulator carry-over from one medium to the next. Although little attention is paid to this phenomenon in the literature it is not credible to assume that the endogenous hormone levels are instantly inactivated at subculture, and play no part in the morphogenic process. With no information with regard to physiologically active levels or rate of metabolism it is only possible to speculate as to the importance of carry-over effects.

In this system, however, detailed anatomical studies can help to clarify the situation. Such investigations (see Chapter 2; Sections 3 and 4) show that the roots and shoots were derived from different, spatially separated parts of the specimen. The roots were produced either from the central region, corresponding to the original hypocotyl, or, at a later stage, from the organised meristematic zones of the terminal calluses; whereas the shoots were produced exclusively from the surface of the callus regions. The bearing of the anatomical studies on the identification of the states of competence and determination are discussed more fully in Chapter 2.

In addition to the extensive studies on organogenesis described above, attempts were made to induce somatic embryogenesis from tisssues of A. thaliana. Unlike the organogenic studies those on embryogenesis were not successful. No embryos were recovered despite considerable manipulation of the culture variables. A few

structures such as those illustrated in Plate 6 were seen on a tissue of Columbia after about 20 days exposure to the first stage medium, but these never developed further.

In addition two pro-embryo-like structures were seen in sectioned tissue, developing within a callus of Enkeim after a similar period in the callus induction medium (see Chapter 2, Plate 2.6). This event was observed only once, however, and there is no proof as to whether these structures were truly the early stages of somatic embryogenesis.

The reasons for the lack of success in this area of the study are not clear but it is possible that concentrating to such a extent on the so called PE tissue produced on calluses of Columbia was a mistake. At the time this tissue appeared to be very promising possessing all the surface characteristics associated with tissue capable of somatic embryo formation. However, the inclusion of a nitrogen source, elevated levels of sucrose, dark and light conditions and various levels and types of auxin and cytokinin failed to cause the production of somatic embryos. Thus, whether this tissue ever had any potential for embryogenesis is called into question. Although it is impossible to prove that somatic embryogenesis will not take place from somatic tissues of A. thaliana it is possible to state that it is not a process which takes place with ease in this species.

This still leaves Huang and Yeoman (1984) as the only report of somatic embryo production from the growing literature on the manipulation of Arabidopsis in culture. As stated earlier, closer examination of their results (Huang, 1985), reveals that the frequency of this reaction and the number of propagules produced was very low, at less than 5%. It would appear therefore that

although organogenesis will occur at reproducibly high frequencies from numerous explants under different culture conditions A. thaliana is not prone to somatic embryogenesis and thus is more akin to tobacco in its morphogenic potential than it is to carrot.

CHAPTER 2

INTRODUCTION

INTRODUCTION

Chapter 1 was concerned with an investigation into the *in vitro* morphogenic potential of *A. thaliana* and the effects of genotype, explant source and culture procedure on regenerative ability. These studies led to the development of simple caulogenic and rhizogenic systems with considerably improved rapidity, synchrony and frequency of response over previously described systems. Such characteristics allow effective examination of the processes behind morphogenesis (Tran Than Van, 1981b; Thorpe, 1980) and in this chapter shoot and root regeneration are investigated in a detailed histological study. This includes the development of the specimen from the time of explanting, the patterns of cell proliferation and callus formation, the generation of new organs and the production and loss of the tissue identified in Chapter 1 as "potentially embryogenic" (PE). First, however, it is necessary to outline the current understanding of the developmental and histological aspects of callus formation and morphogenesis.

Callus Development

Three distinct phases have been identified in the development of callus tissue from the explant material: these are the Lag (or Activation) Phase, the Division Phase and Differentiation Phase (Yeoman, 1970; Aitchison *et al.*, 1978; Warren Wilson, 1984). The wound response induces periclinal divisions just below the damaged surface but this does not persist and cell proliferation will only continue if growth regulators are included

in the culture medium. Yeoman (1970) identifies four types of explant according to this requirement. These are for auxin alone, cytokinin alone, auxin and cytokinin together and, very rarely, the ability to sustain cell division without added plant hormones. This requirement reflects the internal physiology, or inherent cellular state, of the tissue at the time of explanting.

In tissues induced to callus there is a Lag Phase, the exact duration of which is dependent once again on the cellular state of the tissue; young, growing material has a shorter lag time than mature or dormant explants. During this phase considerable cytological changes take place but there is no cell division (Yeoman, 1970).

The Lag Phase ends and the Division Phase begins when a proportion of the cells undergo a synchronous division. This is usually resticted to the peripheral layers of the explant. In embryos and other ontogenetically young tissues the epidermis, and possibly a few layers below it, respond first (Pelisser et al., 1990; He et al., 1990; Vilaplana andd Mullins, 1989; Wang et al., 1990). While in more mature explants, the cambium and tissues located close to it are the usual sites of first division (Sterling, 1951; Smith and Thorpe, 1975; Vasil and Vasil, 1985; Renolds, 1989; Sharma et al., 1990; Kaul et al., 1990). This is thought to start with one cell and is followed by divisions in others around it (Thorpe, 1980). Cell proliferation continues throughout the Division Phase and is characterised by a reduction in the average cell size and the production of meristematic cells. These are isodiametric, with darkly staining cytoplasm, little or

no vacuolisation, have large nulcei and prominent nucleoli and they are considered to be dedifferentiated. The duration of this stage varies and can be prolonged by subculture (Yeoman, 1970) but eventually the average cell size stabilises and the tissue enters the Differentiation Phase.

During this third stage considerable developments take place. Cell division continues but many meristematic cells differentiate to form parenchymatous cells and this increase in cell size results in the first macroscopic signs of callus growth. Changes also take place within the tissue; whereas in the Division Phase cell divisions were restricted to the periphery of the explant and were mostly periclinal producing radial rows of cells (Warren Wilson, 1984; Yeoman, 1970), in the Differentiation Phase meristematic activity proceeds more deeply into the explant to produce nodular and sheet meristems (Yeoman, 1970; Aitchison et al., 1978). The formation of submerged meristematic nodules is a common feature of callus development (Gautheret, 1959; Aitchison et al., 1978) and although commonly recorded the reasons for this are not understood. Yeoman (1970) considers this to "be attributed to some "built in" intrinsic regulatory mechanism which is an inherent property of any given mass of cells".

Three theories have been put forward to explain such pattern formation in plant tissues; Cell to Cell Interaction, Positional Information and Biophysical Forces. The Cell to Cell Interaction Theory described by Meinhardt (1984) stresses the importance of communication between neighbouring cells whereby patterns are set up by the interaction of the autocatalytic

production of an activator and a process that acts antagonistically to its formation. It is considered that such interactions between cells are probably inevitable and it has been shown that such a model can account for the phyllotaxic and branching patterns in plants and, at lower level, the distribution of stomata and hairs on the epidermal surface.

The Positional Information Theory in the original meaning of Wolpert (1971) states that each individual cell has a unique position in the tissue defined by the concentrations and/or polar movement of nutrients or morphogens within its field. The cell interpretates this information and responds to it according to its epigenetic state at that time (Holder, 1979) but unlike the Cell Interaction Theory it does not adjust the signal and so does not affect its neighbours in the process. It is possible, therefore, that the meristematic nodules (or any other morphogenic event) occur at loci where a specific level or combination of morphogens interact with competent cells.

It should be noted, however, that the Positional Information Theory was propossed in order to explain pattern formation in animals and that there is little evidence to substantiate it, especially with regards to plant development.

Plant cells differ from those of animals in that they are bounded by non flexible cell walls which resist changes in cell shape and cell movement; they are thus architectural structures (Sachs, 1978) in which the forces are distributed along the cell

walls. This has led some workers to consider the biophysical forces operating within plant tissues to be paramount in determining pattern formation in plant tissues.

Lintilhac (1974) has shown experimentally that imposing physical stress on a callus induced cell divisions to take place in a regular orientated manner compared to an unstressed tissue. In the latter the divisions were in random directions and produced a disorganised callus. Likewise, Yeoman et al., (1971) disrupted the periclinal divisions in developing tissue by artificially compressing and stressing the material. Lintilhac (1984) states that "the geometrical precision with which stress-mechanical stimuli can be focussed far exceed that possible with molecular diffusates, making it much easier to account for structural adaption at the cellular level". With this theory it is possible to envisage that, as the callus develops into the Differentiation Phase with its associated growth and continued cell division, points, or lines, of stress will be set up which can disrupt the periclinally dividing meristematic cells to induce nodular or sheet meristems.

According to Lintilhac (1984) the major difference between the Biophysical and Positional Information Theories for pattern formation is the role played by the cells' genetic material. For the latter mechanism to operate, the genome must detect, translate and respond to the chemical signals it receives; this must therefore be a highly information rich system. On the other hand response to physical forces within the tissue is thought

to be detected by the microtubules or some structurally similar organelle (Green, 1980), and the only role of the genome is to transcribe the proteins necessary for cell division.

Although the proponents of the Positional Information and Biophysical approaches argue their cases strongly the three theories described above are not mutually exclusive and it is possible that two or more of them operate within plant tissues to determine pattern formation (Lyndon, 1990).

Having entered the Differentiation Phase most calluses consist of a mixture of cell types, the exact nature of which depends on the starting material and the culture medium. The majority of explants are heterogenous consisting of a mixture of cells; for example even simple organs contain cambium, parenchymatous, epidermal, and vascular tissues each with varying degrees of differentiation. These tissues will react differently to the culture system (Street, 1979; Tran Than Van, 1981a; Yeoman and Forche, 1980); those that divide first and have the shortest mitotic cycle will constitute a greater proportion of the resulting callus. A callus is therefore, a dynamic system of cell ratios and types.

This returns us to the importance of the explant source first cited in Chapter 1. To induce a callus with as high a ratio of meristematic and dedifferentiated cells as possible it is usually necessary to choose physiologically juvenile tissues or those containing cambium. These must then be exposed to a culture regime that encourages their proliferation at the expense of the other cell types (Street, 1979).

Once a callus has formed it is often described with terms such as "frothy", "friable", "compact", "wet", and "nodular" these characteristics resulting from the type and degree of differentiation taking place within the tissue. The major part of a mature callus generally consists of thin-walled, vacuolated parenchymatous cells (Thorpe, 1980). Friable tissue results from the differentiation of cells produced by the nodular meristems; these are highly vacuolated, and having little cell to cell adhesion cause the formation of tissue that breaks up easily (Narayanaswamy, 1977; Aitchison et al, 1978). In nodular callus there is less of such differentiation and a more compact tissue is formed. From the practical point of view the morphology of the callus can be an important diagnostic feature allowing the worker to predict the regenerative potential of a particular tissue; for instance friable callus is rarely morphogenic (Narayanaswamy, 1977; Green, 1980; Guierdoni and Demarley, 1988).

The Morphogenic Process

The regeneration of new plant structures takes place from the meristematic regions of the callus tissue. Thus to regenerate new plants the cultured tissue must contain such tissue or cells that will divide to produce them. If the explant is ontogenetically young, and is subcultured before the onset of the Differentiation Phase then regeneration usually takes place form meristematic cells at the surface of the callus. In older calluses

the submerged meristematic centres, or meristemoids, are most often the origin of the regenerants (Maeda and Thorpe, 1979; Murashige and Huang, 1985).

Such meristemoids are initially very small and spherical in shape but soon acquire a polarity when induced to form a new organ (Thorpe, 1980). There is some controversy, however, as to how early the nodule becomes committed to root or shoot formation. They may be initially competent for both and are induced towards a certain fate by the culture system, or they could be determined for a specific fate from their onset (Henshaw et al., 1982). Both roots and shoots can arise from submerged meristems but this is more common for rhizogenesis; most caulogenesis and the vast majority of reported embryogenesis takes place from the meristematic regions at the surface or sub-surface tissue.

One of the major practical questions relating to the exploitation of plant tissue culture systems, is whether or not the regenerating structures originate from one or more cells (Williams and Maheswaran, 1986). This is especially pertinent with regard to somatic embryogenesis and attempts by some workers to equate its developmental patterns to that of the zygotic embryo (Steward et al., 1963). Steward et al., (1958) stated that cell isolation, either physically or by severing of all plasmodesmatal connections, was a requisite for somatic embryogenesis. This was disproved by Konar and Naturaja (1965), and subsequently by numerous other workers, who showed that in the vast majority of cases somatic embryos arise from small multi-cellular groups of meristematic cells; the so called proembryoidal masses (McWilliam et al., 1974;

Jones, 1974; Haccius, 1978; Vasil, 1982). Although this evidence refutes the necessity for cell isolation a number of systems are available to show that somatic embryogenesis can proceed from single cells. This has been demonstrated in Ranunculus sceleratus (Konar et al., 1972) and Freesia (Wang et al., 1990) where embryos arose from single cells of the hypocotyl epidermis and young infloresence respectively.

In organogenesis there is no evidence that shoot formation proceeds directly from one cell, while numerous workers have reported that this regeneration process was also initiated from small groups of meristematic cells (Murashige and Huang, 1985). The question remains, however, as to whether the meristematic centres are derived from one cell or from the products of a number of initials (Jones, 1974; Smith and Thorpe, 1975). Broetjes and Keen (1980) investigated this matter in caulogenesis by studying the frequency of chimeras regenerated from Saintpaulia tissue in which a proportion of the cells were mutated. They concluded that the lack of chimeral regenerants indicated that shoot regeneration must be initiated from a single cell, or from two or three cells which were the daughters of one cell.

Although the question of single or multiple cell origin remains unresolved, Williams and Maharewaren (1986) have formed a hypothesis that would allow both types of regeneration to exist side by side. They consider that if a single competent cell surrounded by non-competent neighbours receives the correct signal(s) it will divide and proceed through the embryogenic process on its own. However, if a group of competent cells are

present which are all at the same developmental state then they can also enter the sequence, and will do so at the appropriate stage for that cell number. In practice the former produces an embryo with a short suspensor-like attachment to the callus while the latter forms a propagule attached to the mother tissue over a much larger area (Haccius, 1978; Williams and Maharewaran, 1986). Despite the differences in the early developmental stages both processes are regarded as being embryogenesis because of the similarities in the later stages which involve the production of all the embryo-specific organs.

The Anatomy of the Regeneration Process

The first visible, histological indication of regeneration is the appearance of organised cell divisions. In shoot regeneration this is seen as the formation of a tunica structure (Tran Than Van, 1981b). Once the apical meristem has been formed it is self regulating, controlling its own cell divisions to form the new shoot. Green (1980) identified this in his studies on bud regeneration in Graptopetalum; he recognises that it is the early events, those involved in the formation of the bulge at the surface of the parent tissue, which are critical and that from then onwards the apex will regulate its own development with simple cell elongation producing the new axis.

The process by which the meristematic cells divide to form such a highly organised structure as the tunica and corpus is not understood but is of great significance. Shoots are always formed with their apices outwards and so the properties of the

callus surface must be important (Green, 1980). Further, to form both a tunica and corpus the cells must have good cell-to-cell contact and perform orientated and coordinated divisions. As already stated friable callus, in which there is poor cell contact, has a low morphogenic potential (Narayanaswamy, 1977; Green, 1980).

The direction of cell division is determined by the orientation of the cell plate, which is in turn determined by the microtubule arrangement (Furuya, 1984; Green, 1980; Lintilhac, 1984). The latter are also involved in controlling the deposition of cellulose in the cell wall which determines the direction of cell extension (Green, 1980). In vivo, cell divisions generally take place according to Errera's Law whereby the new cell wall is formed anticlinally to the direction of organ extension. In a disorganised callus cell division takes place more randomly, and in sheet or nodular meristems both periclinal and anticlinal divisions are present. On subculture to the morphogenic induction medium, however, the pattern of these divisions must change, at least in localised regions, if new structures are to be formed. Furuya (1984) states that physical parameters and growth regulators can effect the cell plate orientation and Maheswaran and Williams (1985) found that BAP, although not required for cell division, increased the frequency of periclinal and oblique divisions in the subcultured tissue.

The Presence and Role of Starch

Since the first report of the presence of starch in regenerating tissues (Thorpe and Murashige, 1968) the role of this polysaccaride in morphogenesis has received intermittent attention. Thorpe and co-workers found starch grains to be present in organogenic tissues and absent in non-regenerating tissues with accumulation preceeding shoot or root formation in the former (Maeda and Thorpe, 1979; Thorpe and Murashige, 1968; 1970; Yeung et al., 1981). Likewise, in a recent study Mangat et al., (1990) describe the build up of starch in Begonia tissues prior to organogenesis and its concentration in the areas of the tissue that would subsequently regenerate. In all of these cases, however, starch was absent, or present in low amount, in the meristematic cells themselves, and it was the cells around the meristematic tissue that were laden with starch (Yeung et al., 1981; Maeda and Thorpe, 1979).

Due to the temporal and spatial appearance of these storage structures starch is considered by these workers to have a causal role in morphogenesis. Whether this is through osmotic control or some other factor is not known.

It is possible, however, that the starch often observed in tissues with morphogenic potential has no such role, and instead just acts as an energy source for the high requirements of the meristematic cells. The dissappearance of the starch during and after the regenerative event could support this conclusion (Yeoman and Forsche, 1980; Stamp, 1987).

The Aims of this Chapter

The work described in this chapter was undertaken to examine in detail the developmental anatomy of the caulogenic and rhizogenic processes. In order to achieve this it was necessary to section and study the cultured tissue from the time of explanting through the cell proliferation phase and transfer to the differentiation medium. This allowed the tissue formation and cell division patterns involved in the organogenic events to be studied and placed in context, spatially and temporally, with each other. The recalitrant type Coimbra was studied in parallel to the highly reactive genotype Einkeim in order to facilitate a possible explanation as to the difference in their morphogenic potential, and examine the reasons behind the recalcitrance of the former. During the study the presence of considerable starch deposits was discovered within some tissues at certain stages in the culture procedure. These were investigated in order to determine their relevance to the regeneration processes.

A secondary aim was to study the tissue indentifed in Chapter 1 was potentially embryogenic, but which failed to differentiate any embryo structures, in an attempt to discover the reasons for this tissue's recalcitrance.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Plant Material

Hypocotyls were excised and subjected to the two-stage culture system developed and described in Chapter 1, Section 4 to induce optimum shoot and root regeneration. Tissue was removed from culture at selected times for fixation and subsequent anatomical examination.

2. Preparation of the Tissue for Anatomical Examination

The chemicals used in the fixation, dehydration, embedding and staining procedures and their respective sources are shown in Appendix 1.

2.1 Fixation

Specimens were picked out of culture and placed in one of the two fixative solutions shown in Table 1 for a minimum of 24 hrs.

2.2 Dehydration

After fixation the tissue was dehydrated by subjection to the series of ethanol and 2-methylpropane-2-ol (TBA) solutions shown in Table 2. If fixed with FAA the tissue entered at step six, thereby omitting the low alcohol stages. Specimens spent at least 1 hour in each stage but could be safely left for longer periods at any step.

Table 1 Solutions used to Fix Cultured Material

1. Formalin/Acetic Acid (FAA)

Glacial Acetic Acid	5 ml.
Formalin	5 ml.
70% C_2H_5OH	90 ml.

O'Brien and McCully, (1981)

2. Chrome/Acetic Acid Fluid (Weak)

10% Aqueous CrO_3	2.5 ml.
10% Aqueous Acetic Acid	5 ml.
Distilled Water	92.5 ml.

Conn et al., (1965)

Table 2 Sequence of Ethanol and TBA Concentrations for the Dehydration Fixed Specimens

Stage	Ethanol	Time (hrs.)
1.	2.5% C_2H_5OH	1
2.	5% C_2H_5OH	1
3.	10% C_2H_5OH	1
4.	20% C_2H_5OH	1
5.	30% C_2H_5OH	1
6.	50% C_2H_5OH	1
7.	TBA 1*	1
8.	TBA 2	1
9.	TBA 3	1
10.	TBA 4	1
11.	TBA 5	1
12.	Sat. soln. of erythrosin in 100% TBA	12
13.	100% TBA	1

* See Table 3 for TBA dilution series.

2.3 Infiltration

The dehydrated and erythrosin-stained tissue was infiltrated with wax by topping up the vials of TBA with paraffin wax chips, and placing in an oven at 70°C. After the TBA evaporated off, two or three changes, of four hours each, with fresh molten wax were performed to ensure complete infiltration.

2.4 Embedding

Rectangular boats were constructed from card and filled with the molten wax. The tissue was transferred to the wax using a pasteur pipette or fine tweezers, and orientated to allow four or five specimens to lie perpendicular to the boat's longitudinal axis.

2.5 Sectioning

Blocks were prepared and 6um sections cut on a Reichert microtome with a Cresson-Original Lung steel blade. The ribbons were expanded by floating on warm water and transferred to slides smeared with Haupts Solution (see Table 4). The slides were then placed to one side and allowed to dry.

2.6 Staining

The dried slides were loaded onto carrier trays and taken through the rehydration/staining/dehydration procedure shown in Table 5.

Table 3 TBA Dilution Series (ml.)

	H_2O	C_2H_5OH (95%)	TBA	C_2H_5OH (100%)
TBA 1	50	40	10	-
TBA 2	30	50	20	-
TBA 3	15	50	35	-
TBA 4	-	50	50	-
TBA 5	-	-	75	25

Table 4 Haupt's Solution

Gelatin	1g.
Phenol crystals	2g.
Glycerol	15 ml.

Procedure: Dissolve the gelatin in 100 ml. of distilled water at 60°C, add the phenol and glycerol, stir and filter.

Table 5 Procedure for Staining Sections with Toluidine Blue

Solution	Time (mins.)
Three passes in 100% Histoclear	3 each
50/50 Histoclear/100% C_2H_5OH	3
100% C_2H_5OH	3
95% C_2H_5OH	3
70% C_2H_5OH	3
50% C_2H_5OH	3
30% C_2H_5OH	3
Dist. Water	3
1% Toluidine blue in dist. water	0.75
Two passes in dist. water	1 each
30% C_2H_5OH	1
50% C_2H_5OH	0.5
70% C_2H_5OH	0.5
95% C_2H_5OH	0.5
100% C_2H_5OH	0.5
50/50 Histoclear/100% C_2H_5OH	1
Five passes in 100% Histoclear	5, or more, each

2.7 Mounting

Slides were picked out of the carriers, shaken free of excess solvent and allowed to dry for about 1 minute. After this time D.P.X. Mountant was dropped onto the slide and a coverslip carefully lowered onto its surface. After drying overnight the sections were ready for examination.

3. Viewing and Photography

The slides were viewed with an Olympus BH-2 microscope with and without the BH-2PC (phase-contrast) attachment, and photographs taken using the Olympus PM-10AD Photomicrographic System. Kodachrome 64 and Panatomic 32 film were used to obtain colour transparancies and black and white prints respectively.

RESULTS

RESULTS

1. Development of the Fixation and Staining Methods

It was found that the standard FAA fixation and staining procedures (O'Brien and McCully, 1981) had to be adjusted in order to achieve acceptable results with the plant material encountered here.

1.1 The Fixation Process

FAA was discovered to be too harsh for the youngest specimen examined. The tissues of fresh hypocotyls prior to explanting and up to explanting plus four days were completely disrupted when fixed with full strength FAA. As it was important to be able to study the anatomy of this starting material efforts were made to find a suitable fixation fluid. Two approaches were taken:

i. attempt to adjust the proportions of the formalin/acetic acid/water in FAA as recommended for delicate tissues by O'Brien and McCully (1981).

ii. adopt a new fixative especially designed for delicate plant tissues.

After considerable manipulation FAA was found to cause rupture and distortion of the cells regardless of its constituent ratios, and therefore a chrome-acetic acid fluid was tried. The weak formulation of this (Conn et al., 1965), designed for the preservation of algae, mosses etc. proved to be markedly more

successful at maintaining the details of these very small and delicate tissues. FAA was, however employed for older tissues as it required considerably less of the time consuming dehydration steps than the chrome-acetic fluid (see Materials and Methods).

1.2 Staining

Initially a staining procedure using safranin and fast green was employed in the hope that this would produce a multi-coloured section highlighting the meristematic regions to the maximum. It was soon apparent, however, that manipulating the relative exposure to each stain in order to achieve this was difficult and the results unpredictable, whilst the long periods of time required in the safranin (1-24 hrs (O'Brien and McCully, 1981)) made the system unwieldy. Toluidine blue was found to be a much simpler and faster system to use and after some experimentation an exposure of only 45 seconds was needed to give good staining. Periods longer than this caused the section to take up too much stain, resulting in the loss of detail.

2. The Anatomy of Callus Formation and Morphogenesis

In order to describe the processes of shoot and root formation and the details of callus development in relation to time in culture, the results of the anatomical investigation are dealt with in the sections listed below.

2.1 Illustration of the major cell types encountered.

2.2 Overall development of the specimen on exposure to the first stage medium from four to twenty days.

2.3 Development of the central region and the regeneration of roots.

2.4 Detailed examination of caulogenesis with comparisons between the responsive and recalitrant genotypes Enkeim and Coimbra.

2.5 Structural changes in the maturing callus and the formation and subsequent loss of the "potentially embryogenic" (PE) tissue in Columbia.

2.6 The presence of embryoid-like structures in callus tissue of Enkeim.

2.7 Shoot regeneration from calluses of the genotype Chisdra after long-term exposure to the first stage medium.

2.8 Illustration of the presence and distribution of starch grains in the developing tissues.

The genotype Enkeim was studied in the greatest detail because it produced roots and shoots in large numbers. The rapidity, high frequency and synchrony of its caulogenic response made it especially suitable for gathering information on the anatomy of shoot formation. These factors made it possible to predict the exact timing of the response and saved undue effort in "capturing" the regenerative events. The majority of the photographs in this Chapter are, therefore, of this genotype,

although others were used where indicated, to investigate certain phenomena; notably recalcitrance in Coimbra and the generation of PE tissue in Columbia.

All sections are longitudinal as experience revealed that cutting in this direction provided greatest information about the developmental state of the tissue.

The condition of the cytoplasm should be considered somewhat objectively because of the inherent tendency of FAA to cause disruption of this material (O'Brien and McCully; 1981).

2.1 **Major Cell and Tissue Types Observed in Culture**

At excision and explanting the hypocotyl consisted of an epidermis and cortex of very large thin walled, vacuolated cells (see Plate 2.1A), with a single celled endodermal layer surrounding the stele. The latter contained two or three protoxylem vessels enclosed in a tissue of numerous small elongated cells, the details of which were not visible. The whole structure was very small, being only 12-16 cells across.

Five major cell/tissue types were identified during the development of the specimen and regeneration of new organs. These will be described here and their further illustration will take place in succeeding sections.

Plates 2.1A - C Systematic Representation of the Distribution of Various Cell Types in Specimens at Explanting and after Four and Twelve Days Exposure to the First Stage Medium

Plate 2.1A T.S. of hypocotyl at explanting showing the epidermis and cortex and endodermis surrounding a thin vascular strand (VS). The xylem elements are enclosed in a narrow sheath of small elongated cells. Scale bar =125 μm

Plate 2.1B L.S. of hypocotyl explant four days after explanting. The specimen is small, about 2um across and only three cell types are present: a strand of protoxylem (1) runs almost the complete length of the tissue and is enclosed in pericyclic tissue, a small amount of parenchymatous tissue (2) at each end and meristematic cells (3) at the central region. The whole specimen is still enclosed in what remains of the original hypocotyl cortex. Scale bar = 750 μm

Plate 2.1C L.S. of specimen after twelve days showing the characteristic dumbell shape with callus material at each end joined by a narrower, non-callused region. The majority of the callus tissue consists of vacuolated parenchymatous cells (2) while its surface is covered by a mixture of loose meristematic cells (4), organised meristematic regions and disorganised non-meristematic cells (5). A strand of protoxylem, two to three, elements thick, runs down the centre of

Scale bar = 750 μm

the non-callused tissue, before branching into the callus ends. Around the xylem is a narrow sheath of elongated cells (1), some of which can be extremely elongated and form a pericycle at the boundary with the meristematic cells of this region.

See Plate 2.1.1 - 2.1.5 for detailed illustrations of these cell types.

Plate 2.1A Hypocotyl Tissue at the Time of Explanting

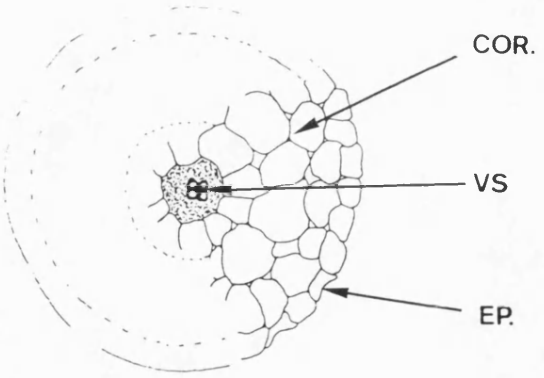


Plate 2.1B Tissue Four Days after Explanting

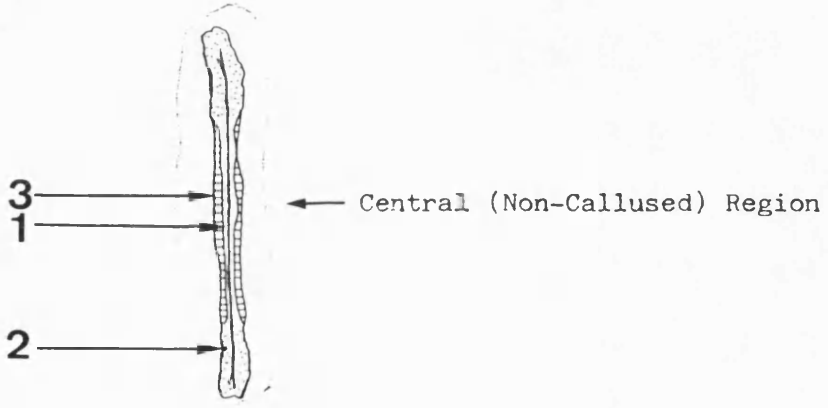
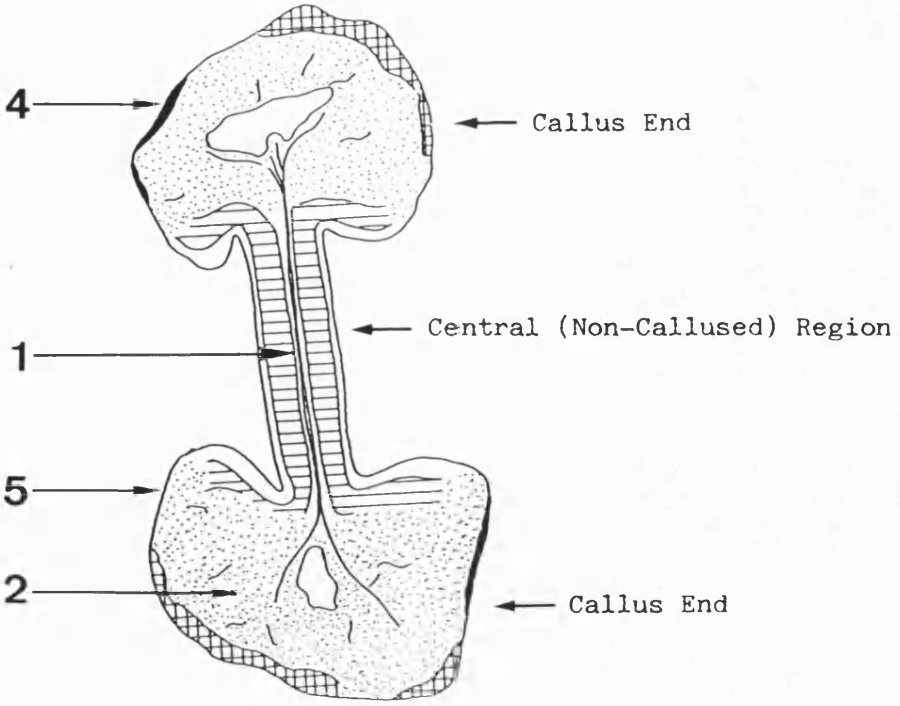


Plate 2.1C Tissue Twelve Days after Explanting



2.1.1 Xylem Tissue

At the time of explanting and until explanting plus two days the xylem tissue consisted of a very fine strand of primary xylem, no more than two to three elements thick, running down the centre of the hypocotyl. These possessed annular or spiral thickening (see Plate 2.3B). After six days the amount of xylem had increased to three to four elements with a mixture of spiral, scalariform and reticulate walls which stained a light torquoise colour. The amount of xylem tissue in the central section did not appear to increase after this time.

The use of phase contrast was very useful in studying the xylem as it stood out clearly from the background tissue when illuminated in this manner. With this type of microscopy it was possible to see that there were no cross walls in the xylem of the central region and that this conducting tissue consisted exclusively of elements.

The xylem was not restricted to the core of the central, non-callusing tissue but was also seen to branch out of this region and spread through much of the callus at each end of the dumbell from the six day stage onwards (see Plates 2.1B and 2.4A). The majority of the xylem in the callus was in the form of tracheids but due to its three-dimensional branching it was difficult to follow the pattern of the xylem in the callus. However, it appeared that the majority, if not all, of the xylem in this region was continuous with that of the central region.

2.1.2 Elongated Cells

Associated with the xylem tissue were cells with varying degrees of elongation. These formed a narrow pericyclic sheath around the xylem in the central region, with greatest elongation occurring at the outer edge of this cylinder (see Plates 2.1C-1 and 2.3). At its most extreme the cells were 20-25 times longer than they were wide with thick walls which had the tendency to stain a dark red colour. Inward from these the cells were less elongated, varying between five and ten times greater longitudinally than transversly, having tapered or perpendicular end walls and lightly staining cell contents with the occasional visible nucleolus.

Like the xylem tissue the thin walled elongated cells were also found in the callus ends, sometimes in close association with the former but often seemingly remote from it. Groups of these cells formed swirls within the callus and were often seen near the surface of the tissue (see Plate 2.1C-4). Whether the elongated cells of the callus and central regions were continuous and of a common origin was difficult to determine from the sections observed.

2.1.3 The Callus Tissue

The callus tissue present at each end of the "dumbell" consisted of a mixture of cell types. Already described are the xylem and elongated cells. The majority of this tissue, however, consisted of cells with differing shapes and sizes with thin walls, little visible cytoplasm and small nuclei. They were loosely

packed together with some intercellular spaces and in most cases they took on a pale pink colour from the toluidine staining (see Plates 2.1C-2 and 2.4A). The cells of this region therefore fulfilled the classic description of parenchymatous tissue.

2.1.4 Meristematic Cells

Meristematic cells were present in all the specimens examined. These were identified by their relatively small size, deeply blue/purple stained cytoplasm, very large nucleus with large dark blue or red nucleolus and often by indications of recent division. Meristematic tissues were seen in three different locations.

2.1.4.1 Meristematic cells in the central region

In the central region of the specimen, exterior to the xylem and its sheath of elongated cells a cylinder of meristematic cells developed. At the four to six day stage these formed a layer about six cells thick in which they were packed together tightly and in a highly ordered manner. A distinctive protodermal-like layer was present (see Plates 2.1C5 and 2.3J) which formed a perfectly smooth, if undulating, surface to this region. This was responsible for the non-callused appearance of this part of the specimen.

Under the protodermal-like layer the cells were packed with slightly less order but intercellular spaces were never seen. Divisions were visible in both the periclinal and anticlinal planes

Plates 2.1C 1 - 5 Illustration of the Major Cell Types Encountered in the Tissue of Developing Calluses

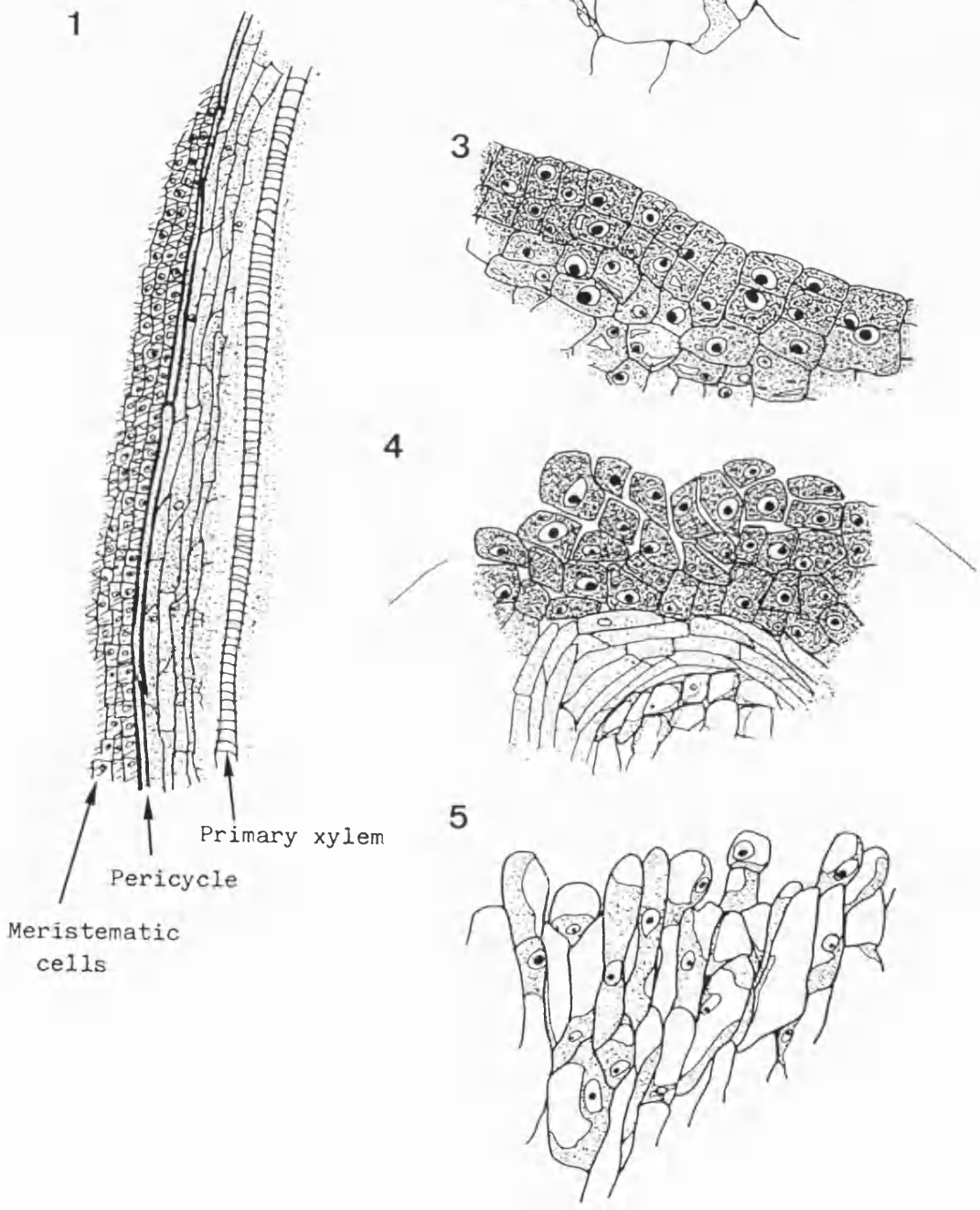
Plate 2.1C 1 Elongated cells found in a sheath surrounding the xylem elements and separating them from the meristematic cells of the central region. A uniseriate layer of elongated pericyclic cells with thickened walls was often seen at the extremity of the sheath.

Plate 2.1C 2 Vacuolated cells with occasional intercellular air spaces. This cell type constituted the majority of the callus ends.

Plate 2.1C 3 Meristematic cells at the surface of the central region in young specimens (four to six days after explanting).

Plate 2.1C 4 Unorganised, highly meristematic cells at the surface of the callus tissue. Directly beneath these are some elongated cells which were often associated with the former. Below these are the parenchymatous cells.

Plate 2.1C 5 Non-meristematic cells at the surface of the callus tissue. These are large and often ruptured by the preservation techniques used here.


1
2
3
4
5
Primary xylem
Pericycle
Meristematic
cells

(see Plate 2.3D). The cells nearest the vascular strand were generally larger and more vacuolated than those at the periphery (see Plate 2.3C).

As the specimen aged the meristematic tissue in the central region changed but this will be discussed in Section 2.3.

In most cases the meristematic tissue of the central region extended some distance into the basal end of the callus tissue (see Plates 2.1B and 2.2D)

2.1.4.2 Meristematic cells in the callus tissue

In addition to this band of meristematic tissue which extended into the callus from the central region, meristematic cells were found in the callus tissue itself. In specimens six to ten days old these were restricted to a disorganised layer two to four cells thick situated at the surface of the callus (see Plates 2.1C-4 and 2.4B). The proportion of the callus covered by these cells varied with the genotype and the time exposed to the callus induction medium. The importance of these cells with regards to caulogenesis will be dealt with in Section 2.4.

Meristematic cells at the callus surface were most numerous at the six to eight day stages and were distinctive in their deeply staining cytoplasms, large nuclei and large red-staining nucleoli. Unlike those of the central region, they were not packed tightly nor were they present in any order; instead they were of varying shapes and sizes, often with rounded walls and only in loose association with each other.

2.1.4.3 File meristems

The presence of the loose meristematic surface cells was maximal at the six to eight day stage. After this time increasing proportions of the callus surface were occupied by non-meristematic cells (see Plate 2.2C) and by large, solid, organised, meristematic structures.

The latter were present in the form of file meristems and consisted of numerous files of cells packed and stacked together with great uniformity and order. Such tissues formed domed structures (see Plate 2.5B) the detailed structure and development of which will be dealt with in Section 2.5.

2.1.5 Non-Meristematic Cells at the Callus Surface

In addition to the meristematic cells - either present as a loose disorganised layer or as a file meristem - part of the callus surface consisted of non-meristematic cells. Like the meristematic cells the proportion of the surface occupied by vacuolated tissue varied between individuals, genotypes and culture time.

The non-meristematic cells were mostly elongated to some extent with rounded walls at the extreme exterior (see Plate 2.1C-5). Transverse divisions were common forming short filaments protruding from the callus surface. They had thin walls, small nuclei and were packed together loosely with intercellular spaces being common. Preservation was poor in many cases indicating their thin walled, highly vacuolated nature. The size of these surface

cells varied with some massive examples being seen on Coimbra (see Plate 2.4J). Intact cells had some lightly staining cytoplasm and small nuclei.

2.2 Overall Development of the Specimens from Four to Twenty Days Culture on the First Stage Medium

Plates 2.2A - 2.2G illustrate the growth and development of the tissue on exposure to the callus induction medium designed in Chapter 1 from day four to day 20. Details of tissue younger than this will be examined in Section 2.3. Although there was some variation in the size and developmental stage of the tissues at any given time this was found to be minimal, and the general patterns described here were highly reproducible.

Four days after explanting the developing specimen was only about 20μm thick and still enclosed in the cortical tissue of the original hypocotyl. It is not possible to tell whether the cortical tissue was still alive at this time, as the fixative had caused considerable damage to its cells, but there was no evidence of cell division in this tissue and it is assumed in the following account that it took no part in the development of the callus or central regions. These seemed to be derived entirely from the stelar tissues of the hypocotyl. In the latter region considerable development had taken place and an area of darkly stained

Plates 2.2A - H Sections through Developing Specimens of the Genotype Enkeim Cultured Continuously on the Callus Induction Medium

Plate 2.2A Section through a specimen four days after explanting showing a sheath of meristematic cells (MS) around the vascular tissue in the central region. The tissue at either end is less darkly stained and the whole specimen is still enclosed within the original cortical tissue (C) of the hypocotyl.
Scale bar = 500 µm

Plate 2.2B Section showing development after six days. Both ends have grown considerably to produce calluses with a rough disorganised surface. The outer edge of the callus is covered in a thin layer of cells that have stained more heavily than the interior.

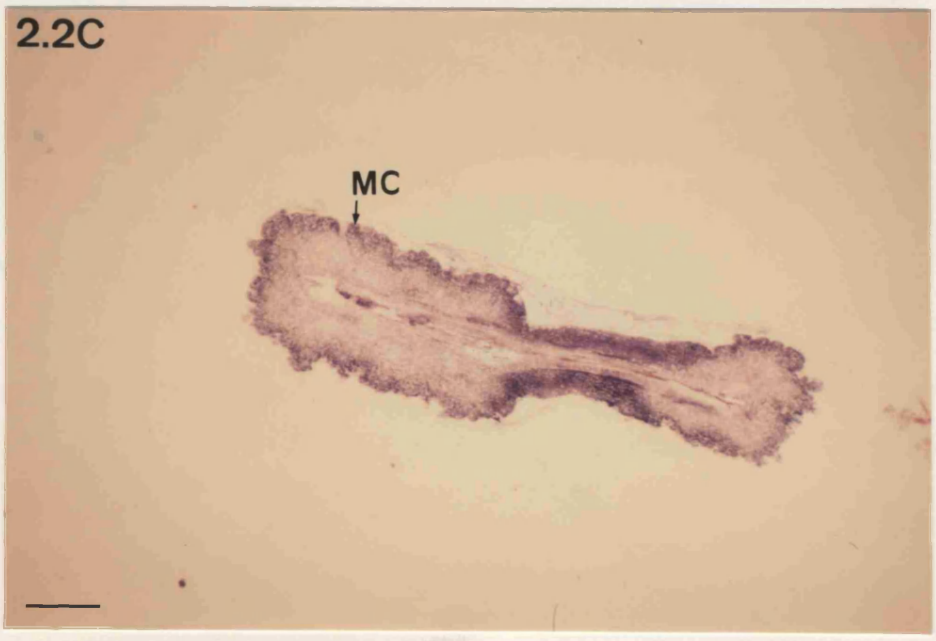
The central section has increased in girth and in contrast to the callus at each end has a smooth, undulating surface.
Scale bar = 500 µm

Plate 2.2C An eight-day old specimen showing continued growth of the callus ends and the presence of a distinctive layer of meristematic cells (MC) at its surface.
Scale bar = 500 µm

2.2A

2.2B

2.2C



meristematic cells was visible forming a sheath around the vascular strand. At either end the tissue was much lighter in colour and slightly greater in width.

By day six (Plate 2.2B), the tissue had grown considerably and the bi-lobed or dumbell shape, so characteristic, even at the macroscopic level, was evident. The central region had expanded considerably due to increased cell number and had a very smooth surface composed of darkly stained meristematic cells on either side of a lighter core. In contrast the callus at both ends consisted of more lightly staining material with a rough, irregular surface. A large proportion of the callus surface was covered in darkly stained, meristematic cells.

This pattern of growth continued until the eight day stage. Plate 2.2C shows the increase in size by this time and illustrates well the presence of meristematic cells over most of the callus surface. A cavity is also visible at the centre of each callusing end. This was seen in almost all the sections from this stage onwards but it is suspected that these were an artifact of the histological procedure.

The central region had also grown since day six. In addition to its increased girth, however, there was a change in the structure of the tissue. By this stage the meristematic tissue of this region was no longer outermost, but instead it was surrounded by a layer of lightly stained tissue and finally by an epidermis-like layer. The latter still formed a smooth, organised surface to the central region and with its intermediate staining it

was quite distinctive. Finer details of this development, which is evident in all specimens older than six days, will be described in Section 2.3.

Changes continued to occur with exposure to the first stage medium. Increasingly, instead of being covered with meristematic cells the surface now consisted of areas of loose, non-meristematic cells and regions in which the meristematic tissue was many cells thick. By explanting plus 10 days (Plate 2.2D) this patterning was distinctive, as was the presence of bands of meristematic tissue at the base of the callus ends. These were continuous with the meristematic tissue of the central region and like the latter had a layer of vacuolated, non-meristematic cells to the exterior. In this case however they did not form an epideral-like layer as in the former.

By day 12 (Plate 2.3E) these trends had continued and accompanying the continued growth of the callus at each end, there was the development of distinctly solid regions of meristematic tissue at the surface of the callus. These are prominent at both ends of the illustrated example. The meristematic areas became more substantial in size and were many cell layers deep. In contrast to the younger tisssues (6-8 days) a smaller proportion of the callus surface was covered by the loose meristematic cells.

Increasing formation of solid bands of meristematic tissue in the callus ends continued through the 14 and 16 day stages. By this time the dark bands situated just below the tissue surface were almost continuous around the whole specimen and there was little evidence of the narrow layer of disorganised

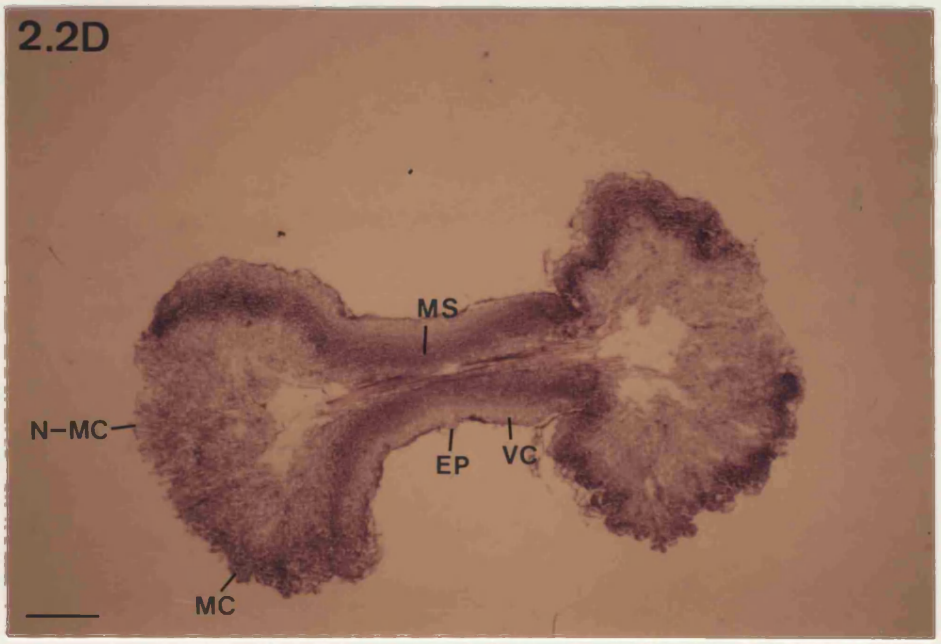
Plate 2.2D Development after 10 days showing much enlarged calluses at each end and a number of structural changes.

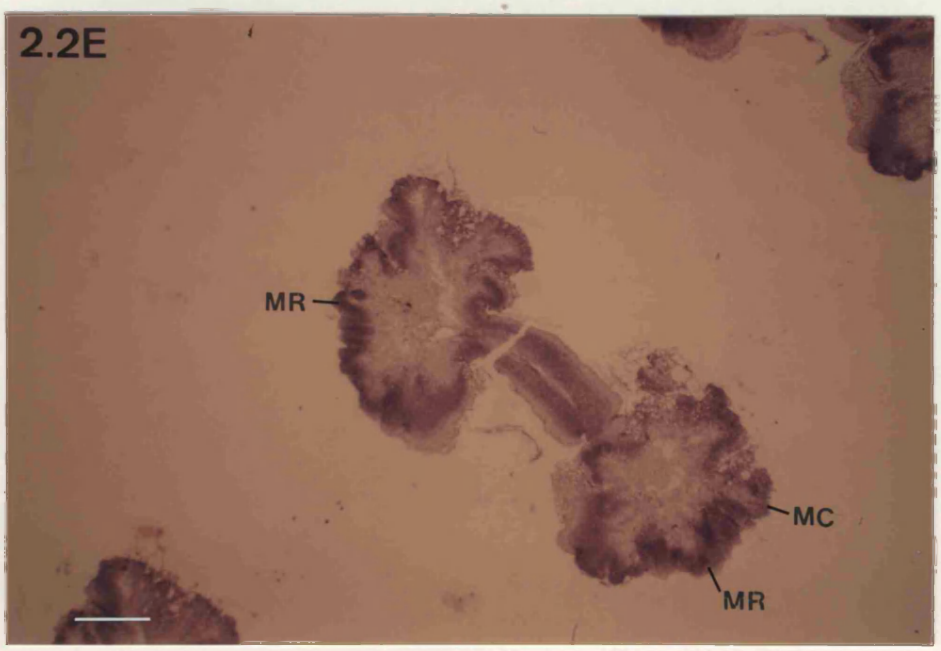
In the central section, in addition to the vascular strand, three tissue layers are visible. These are inwards; an epidermis-like layer (EP), a layer of vacuolated cells (VC) and the meristematic sheath (MS).

The callus tissue has also changed, now consisting of loose meristematic cells (MC), loose non-meristematic cells (N-MC) and bands of meristematic material spreading from, and continuous with, that of the central region.
Scale bar = 500 μm

Plate 2.2E Section through a 12-day old specimen. Development has taken place to form discreet darkly stained regions (MR) at the callus surface. These are composed of many layers of meristematic cells. Only small areas of the surface are now covered in loose disorganised meristematic cells (MC).

The band of meristematic cells extending from the central region into the basal ends of the calluses is clearly visible, as is the layer of vacoulated cells on its exterior.
Scale bar = 1 mm


2.2D
MS
N–MC
EP
VC
MC


2.2E
MR
MC
MR

meristematic cells, so distinctive at the younger stages. There were, however, considerable areas of covered with non-meristematic cells.

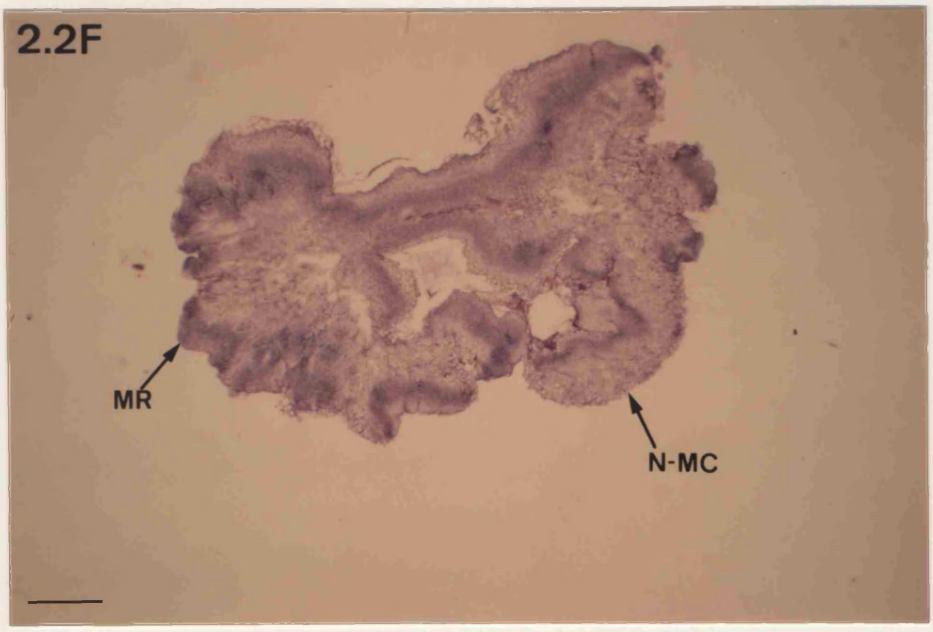
Plates 2.2F and 2.2G both illustrate the gradual loss of the bi-lobed shape with continuing time in the first stage medium. The growth of the callus ends towards each other, seen in Plate 2.2G, and/or the break-up of the structure of the central region, seen in Plate 2.2F assured this.

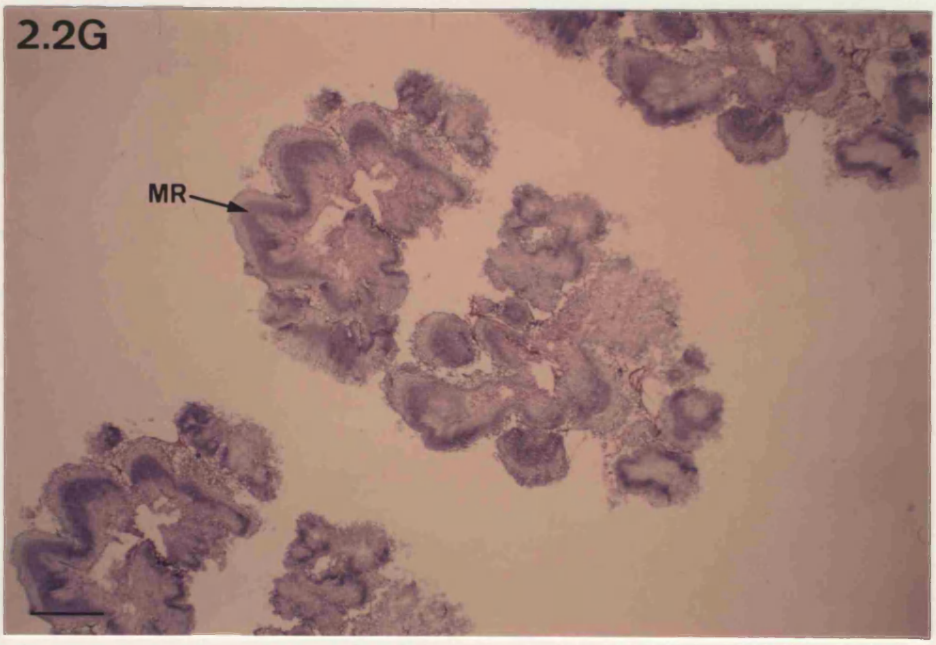
After 20 days culture in the first stage medium the tissue was relatively very large and was difficult to section effectively. The "dumbell" structure gave the plant material an easily recognisable longitudinal axis by which to orientate the tissue for sectioning. This had, however, been lost by the 20 day stage, making it difficult to identify the true longitudinal axis. Further, the callus became contorted and twisted as it developed and with many raised surfaces it was almost impossible to obtain a section through the whole tissue. Plate 2.2G is, however, a reasonable representation of the callus at this age. The section is split in two as a consequence of the connecting tissue being present in a different plane.

The callus consisted of various regions either with no meristematic tissue or having bands of darkly stained cells situated just below the surface. The detailed composition of these dome-shaped structures will be examined in Section 2.5.

Plate 2.2F L.S of a 16 day old specimen showing the meristematic tissue of the central and callus regions to be continuous in parts. The callus surface consists of solid meristematic regions (MR) or areas of non-meristematic cells (N-MC).
Scale bar = 1mm

Plate 2.2G After 20 days the tissue has lost its bilobed shape and is a complicated mixture of darkly stained meristematic (MR) and non-meristematic regions.
Some of the tissue shows considerable organisation.
Scale bar = 1mm


2.2F
MR
N-MC


2.2G
MR

2.3 Development of the Central Non-Callused Region and the Regeneration of Roots

These two subjects are dealt with together because it was from the central region of the specimen that the majority of root regeneration took place. Some roots were produced by the callus tissue in older specimens but the large scale, synchronous rhizogenesis was restricted to the central region (see Chapter 1, Section 4.2).

2.3.1 Development of the Young Explant

At the time of explanting and for the first 24 hours the hypocotyl consisted of an epidermis, cortex and vascular strand as shown in Plate 2.1A and Plate 2.3A with the whole structure being very small in cross section. The xylem was annularly or spirally thickened and surrounded by a pericyclic sheath of very narrow, elongated cells which had a mixture of transverse and tapered end walls. There was very little visible difference between this tissue and the original hypocotyl except that the sheath of elongated cells had taken on a deep blue/purple colour.

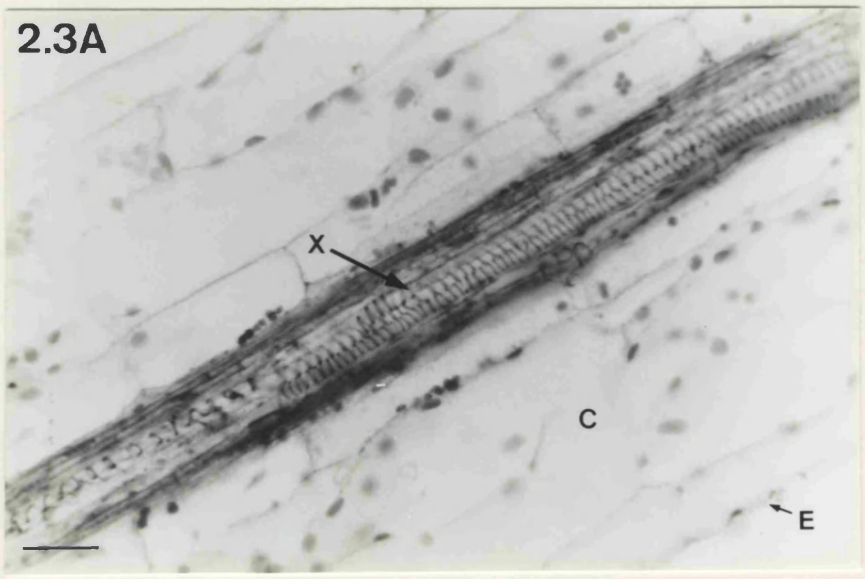
After two days (Plate 2.3B), however, a dramatic change had taken place whereby the elongated cells in the stelar region had expanded transversly and become meristematic. This was evident by their darkly stained cytoplasm and large nuclei. Divisions were taking place in both the periclinal and anticlinal planes.

Plates 2.3A - D Development of the Central Region and the Regeneration of Roots

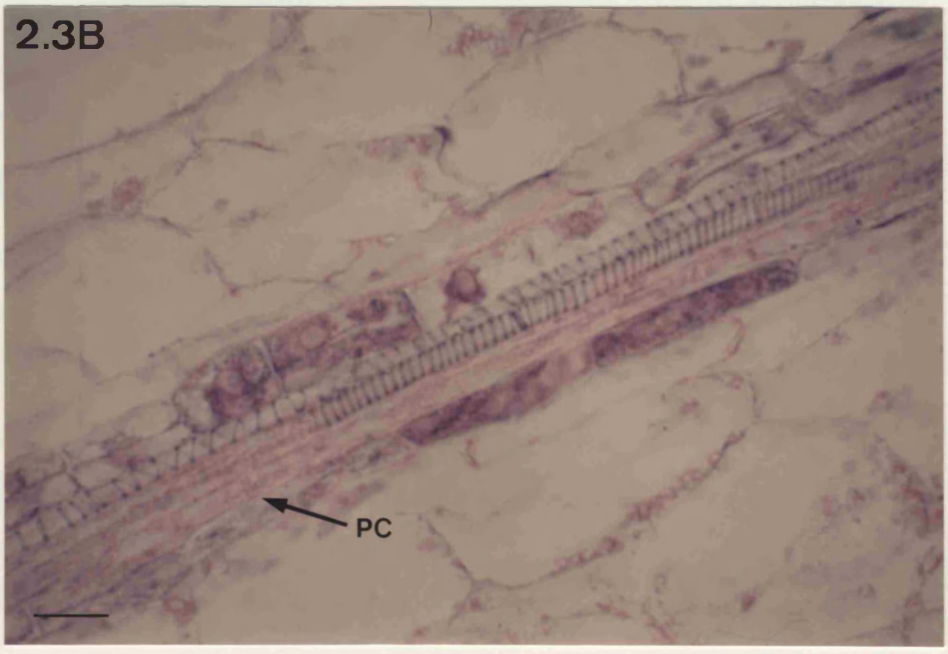
Plate 2.3A Section through a hypocotyl one day after explanting. The total width of the organ is represented in this photograph with the epidermis(E), cortex(C), possible endodermis and vascular strand all visible. The annularly and spirally thickened xylem elements (X) are surrounded by a sheath of narrow, elongated procambium and/or pericyclic cells. These have a mixture of tapered and transverse end walls.
Scale bar = 65 μm

Plate 2.3B Detail of the vascular strand and procambium tissue at explanting plus two days. Some of the elongated procambium/pericyclic cells are still visible (PC) but most have enlarged transversely becoming heavily stained with large nuclei. There is evidence of both periclinal and anticlinal divisions by these cells.
Scale bar = 50 μm

2.3A



2.3B



Massive cell division took place in both of these directions over the next two days to produce a cylinder of meristematic cells five to eight cells deep around the vascular strand. These divisions were not random and no callus was formed; instead they were coordinated and organised to produce a tightly-packed tissue with an outer protodermal-like layer (see Plates 2.3C and 2.3D). This imparted a smooth organised appearance to this region of the specimen. These cells were highly meristematic, being small, densely stained and non-vacuolated, with large nuclei and dark nucleoli. In addition, there were many indications of recent divisions in this tissue.

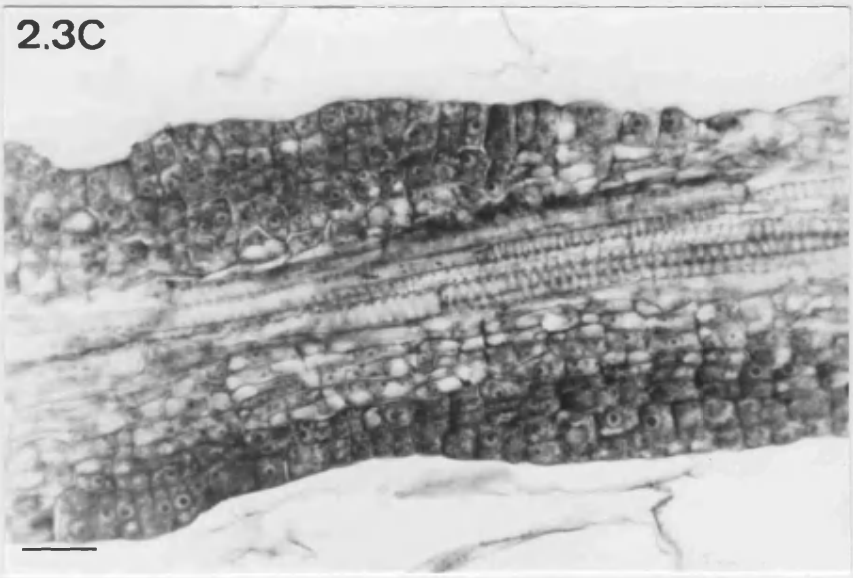
Transfer at this stage, even to a medium supplemented with relatively high levels of cytokinin (10^{-6}M BAP) induced root formation from 100% of the replicates, see Chapter 1, Figure 12. This was a highly synchronous and rapid event with multiple roots being produced within two days of subculture. In order to study the anatomy of this organogenic event sections were obtained from material 12, 24 and 48 hours after subculture to the second stage.

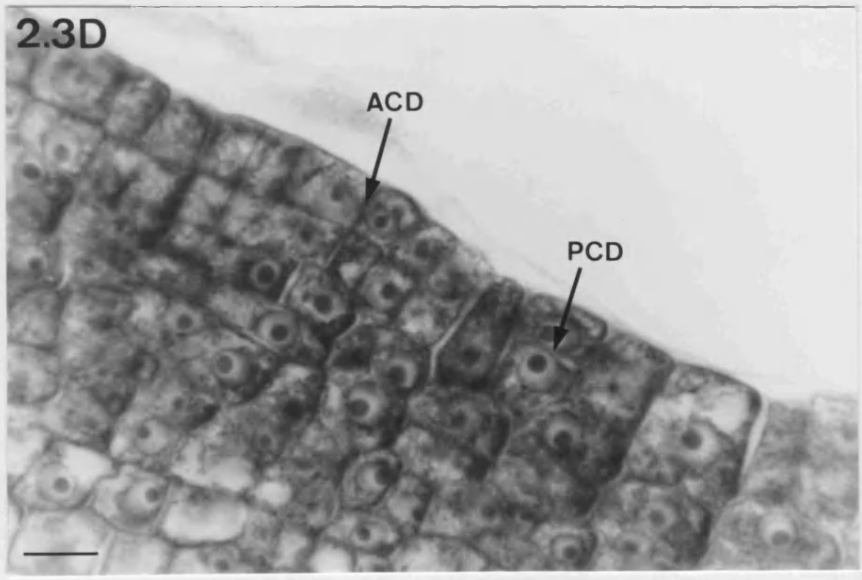
Root formation took place from the meristematic tissue of the central region as a whole. Development was rapid and within 24 hours of transfer the young roots were clearly visible (Plate 2.3E) and by 48 hours provascular tissue connections were differentiating from the new apices (Plate 2.3F).

The exact origin of the roots was not clear, but large areas of the meristematic tissue were involved. What had been an undulating surface was quickly transformed into a numerous apical meristems. Plate 2.3G shows a group of cells at 12 hours after

Plate 2.3C Massive cell division has produced a sheath of small meristematic cells around the xylem by day four. The divisions have been well ordered so as to produce a tissue of tightly packed cells with a smooth organised protodermal surface. The cells become larger and more vacuolated nearer the vascular tissue.
Scale bar = 65 µm

Plate 2.3D Detail of the meristematic cells at day four, showing their meristematic characteristics (heavily stained cytoplasm, large nuclei, distinctive nucleoli and micro-vacuoles) and the formation of an epidermal-like layer at their exterior. There is evidence of both anticlinal (ACD) and periclinal (PCD) divisions.
Scale bar = 30 µm

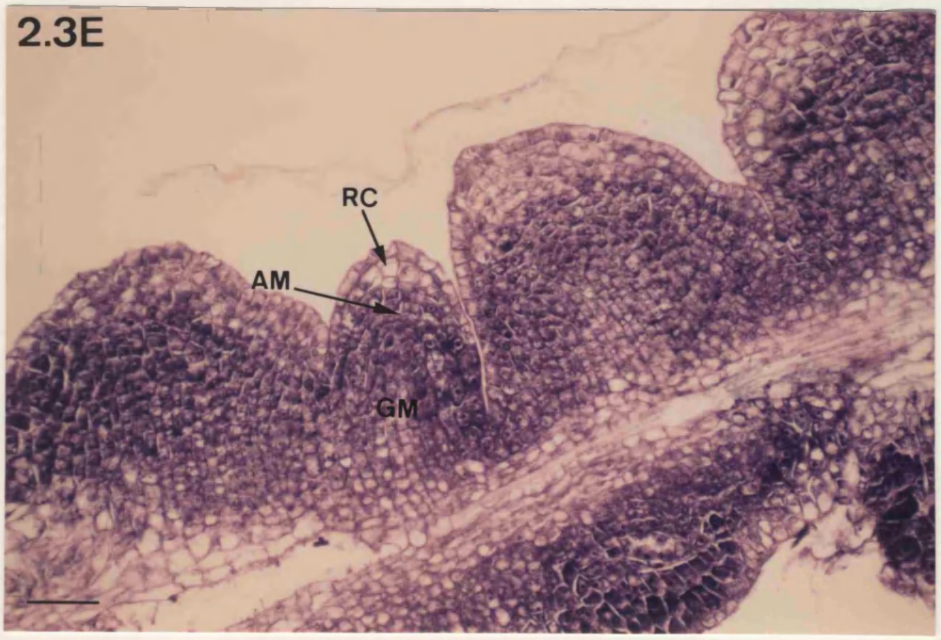

2.3C


2.3D
ACD
PCD

Plates 2.3E - H Root Formation from the Central Region

Plate 2.3E Young roots forming from a specimen after four days in callus induction medium and one in the shoot induction medium. Distinct root structures are visible with vacuolated cells present in the region analagous to the root cap (RC), an apical meristem (AM) and ground meristem (GM).
Scale bar = 100μm

Plate 2.3F Section of the central region two days after subculture. Numerous roots are developing the most mature of which have provascular connections (PV) with the mother tissue.
Scale bar = 200μm


2.3E
RC
AM
GM

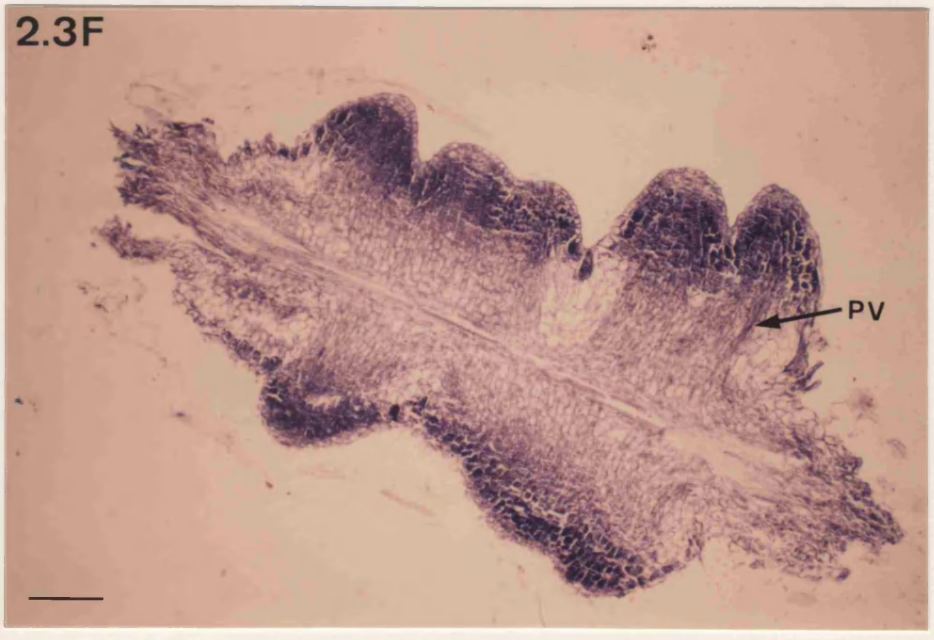
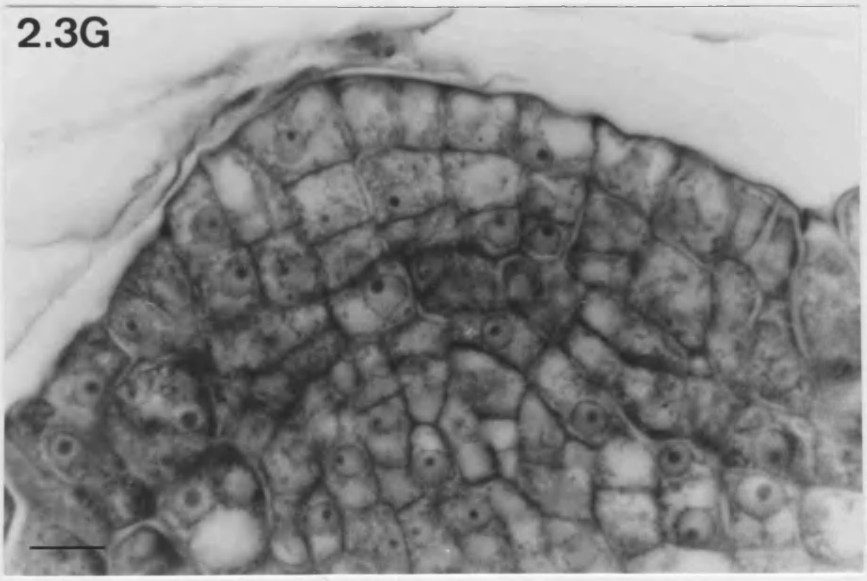
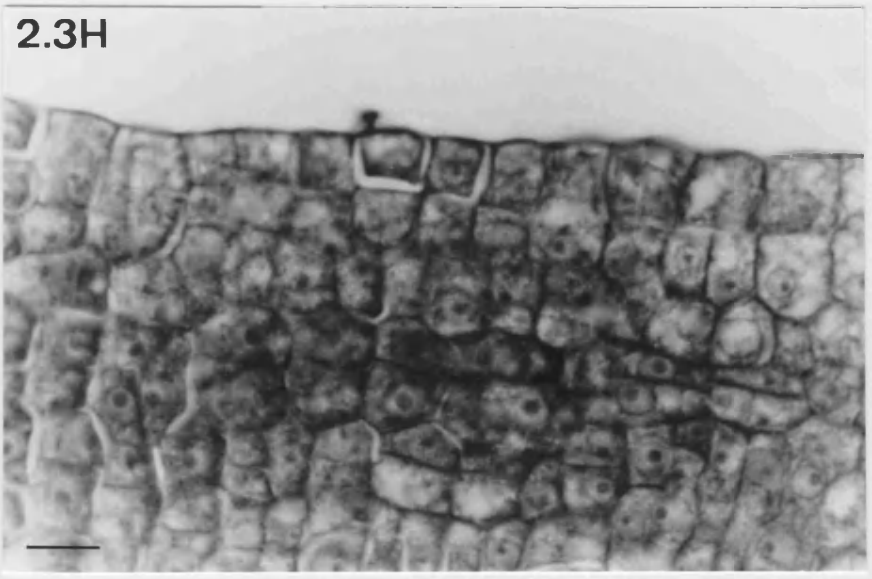

2.3F
PV

Plate 2.3G Detail of a region of the surface 12 hours after transfer to the second stage. This shows a localised bulge in the tissue that could be a young root primordium.
Scale bar = 30μm

Plate 2.3H Meristematic cells of the central region under high magnification. Such localised areas of periclinal divisions might lead to the situation illustrated in Plate 2.3G.
Scale bar = 30μm

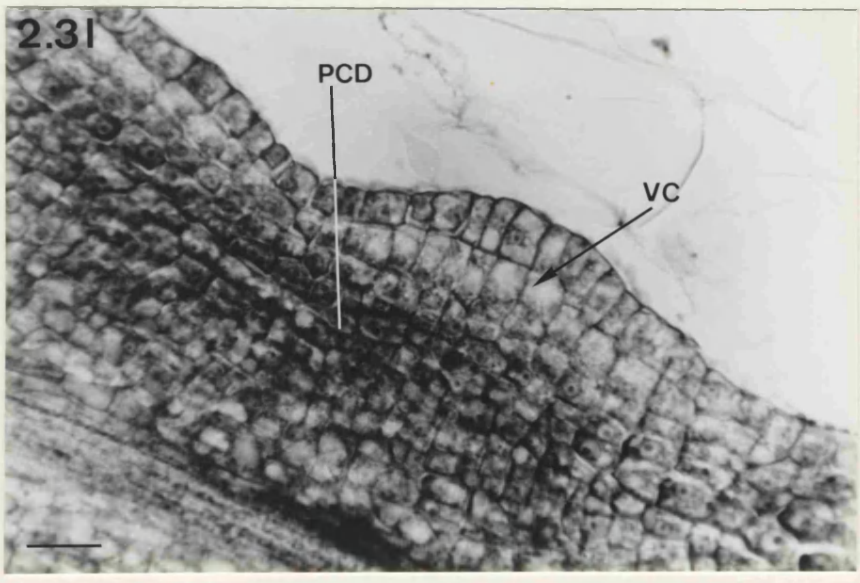

2.3G

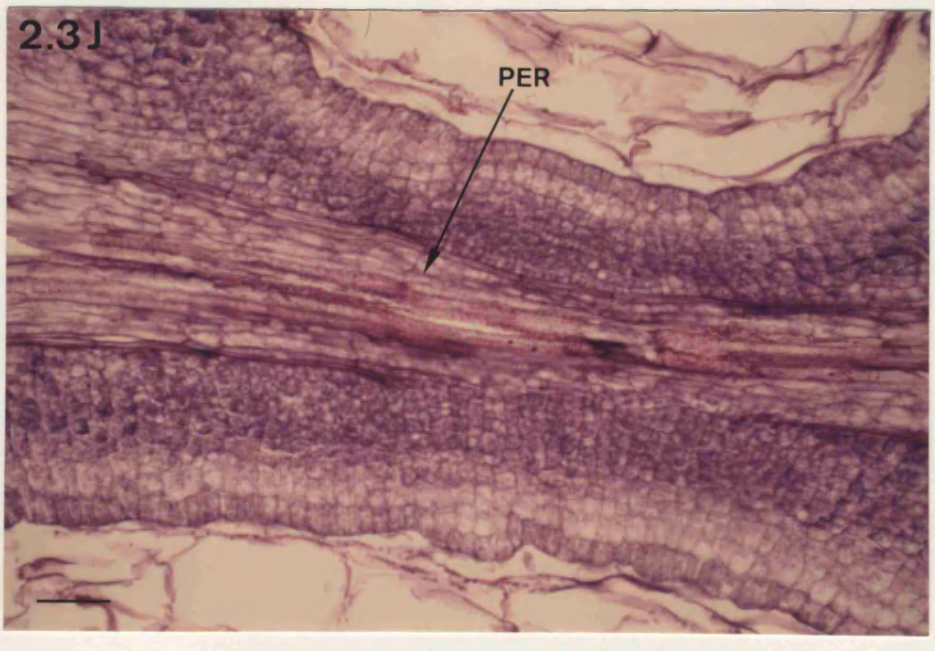

2.3H

Plates 2.3I - J Development of the Central Region from Six Days after Explanting Onwards

Plate 2.3I L.S of the central region after six days on the callus induction medium showing the development of a vacuolated cell layer (VC), two to three cells thick, between the meristematic cells and the epidermis-like layer. Recent periclinal divisions (PCD) are visible in the meristematic tissue.
Scale bar = 125μm

Plate 2.3J The developmental state after 10 days on the callus induction medium. The intermediate staining of the epidermal-like layer distinguishes it from the pale vacuolated cells beneath it. Also visible in this section is the presence of uniseriate, extremely elongated cells forming a pericyclic region (PER) around the stelar tissue.
Scale bar = 100μm


2.3I
PCD
VC


2.3J
PER

subculture and what appeared as a slight swelling on the surface of the meristematic tissue, under high power turned out to be possibly a young root primordium. In Plate 2.3H localised periclinal divisions are seen which might be responsible for the creation of such bulges and subsequent root regeneration.

Figure 12, Chapter 1, illustrates that, if subculture was delayed until six days or more after explanting, rhizogenesis decreased dramatically. Further, roots were not produced from the central region after this time. Plates 2.3I and 2.3J show the changes that had taken place in the structure of this tissue by the six to eight day stage.

Both of these photographs illustrate clearly that a layer of cells, two to three thick, immediately below the outer layer had become vacuolated. These had expanded transversely and possessed a pale, granular cytoplasm with smaller nuclei than the meristematic cells below them. In addition the protodermal-like cells had been replaced by an epidermal-like layer with an intermediate degree of staining. Its nuclei are now a pale blue colour with small dark nucleoli, rather than colourless with red nuclei, as in the highly meristematic cells of the four-day stage.

The number of cells contributing to the vacuolated layer increased with increasing time in culture but the ordered cell packing of this region was not seen to break up until the 16 to 20 day stage.

Plate 2.3J also shows the presence of the extremely elongated cells described in Section 2. These uniseriate cells formed the outer boundary of the vascular strand in the region usually occupied by the pericycle and internal to them are lesser elongated cells and xylem vessels.

2.4 Shoot Regeneration and Development of the Callus Tissue

Shoots were never formed from the central region but always from the callus ends. Maximum caulogenesis took place if the tissue was transferred to the second stage after six or eight days exposure to the callus induction medium. Subculture at times later then this reduced competence for shoot regeneration until it was eventually lost altogether (see Chapter 1, Figure 9).

In genotypes with a high caulogenic potential, such as Enkeim, multiple shoot regeneration was seen with the naked eye four to six days after subculture. The purpose of this section is to investigate the histology of the callus tissue at its time of maximum competence, and the changes that take place immediately after subculturing until the time of macroscopically visible shoots.

2.4.1 Characteristics of the Caulogenically Competent Callus

Plates 2.2B and 2.2C illustrated the presence of deeply stained cells around the outer surface of the callus at six and eight days after explanting. Further evidence of this phenomenon

is shown in Plates 2.4A and 2.4B. Under this higher magnification these cells are clearly seen to be meristematic, having the characteristic small size, dark cytoplasm, large nuclei and nucleoli of such cells. They are present as a disorganised layer no more than four or five cells thick at the periphery of the callus tissue. Internal to them can be seen the pinkish coloured, thin walled, vacuolated parenchymatous cells that constitute the bulk of the callus.

In some specimens elongated cells in the callus tissue were associated with the meristematic cells on the surface. These were often seen immediately below the surface, abutting the meristematic cells.

2.4.2 Shoot Regeneration from Enkeim

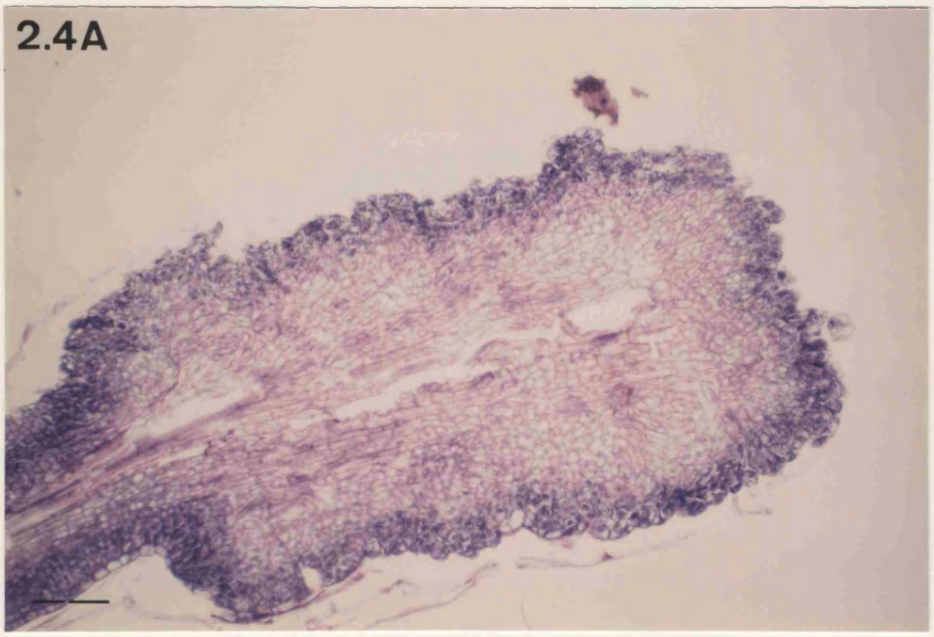
New apical meristems always arose at the surface of the callus but the time and manner by which this took place was difficult to record. No organisation was observed in material at 12 hours after subculture, and it was not until a total of 24 hours on the shoot induction medium that the first indications of regeneration were visible.

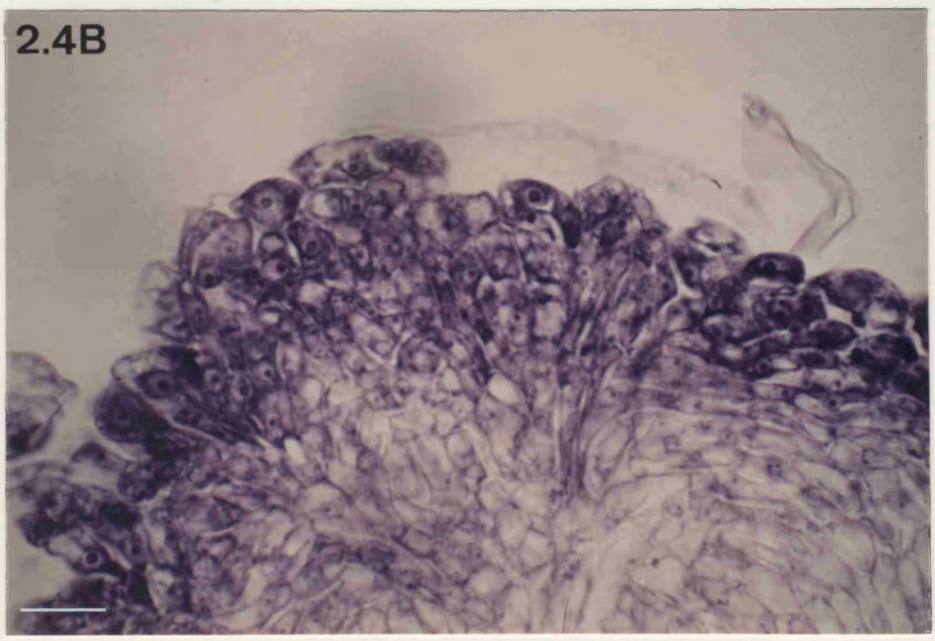
Plate 2.4C shows what could be the formation of a tunica-like structure at this time. It appears that a group of about four cells have formed a domed, uniseriate layer in which the cells are in intimate contact with each other. This contrasts with the disorganised nature of the surrounding meristematic cells.

Plates 2.4A – C Anatomy of the Caulogenically Competent Callus

Plate 2.4A Tissue after exposure to the callus induction medium for eight days showing a distinctive layer of darkly stained disorganised meristematic cells around the outer edge of the callus. This section also illustrates the penetration of the xylem elements into pink stained parenchymatous callus region.
Scale bar = 200μm

Plate 2.4B Detail of the callus edge to show the meristematic state of these cells. They are one to four cells deep and have no ordered structure. The irregular size and shape of the larger, relatively vacuolated cells of the callus tissue is also visible.
Scale bar = 50μm

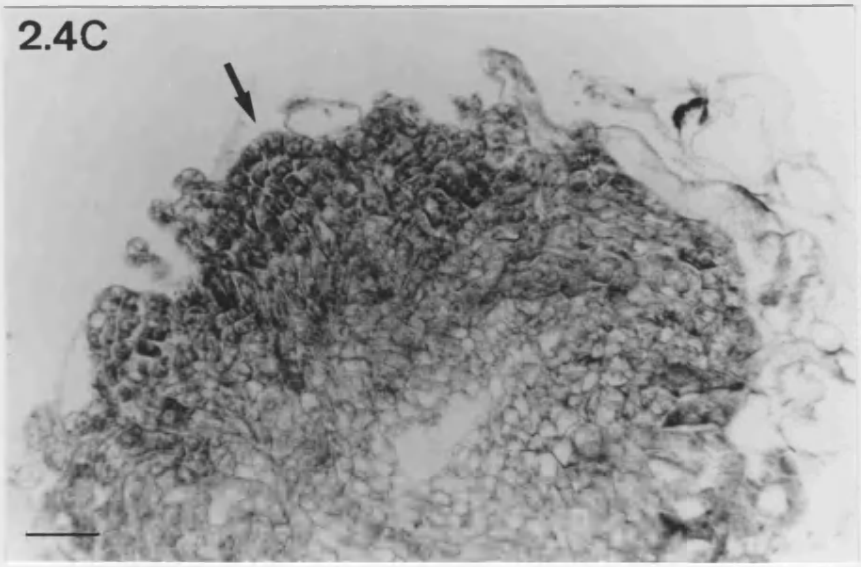

2.4A


2.4B

Plates 2.4C - G The Anatomy of Shoot Regeneration

Plate 2.4C L.S of tissue after six days on the first stage and one day on the second stage medium. The earliest sign of organisation were seen 24hrs. after transfer to the second stage. A group of four to five cells has divided to form a curved, uniseriate layer resembling a tunica structure (arrowed). These have intimate cell to cell contact, which contrasts with the disorganised state of the surrounding meristematic cells.
Scale bar = 125μm

Plate 2.4D A young meristem formed after eight days on the callus induction and two days on the second stage medium. It is small, about 30um thick, and has a one-layered tunica.
Scale bar = 65 μm


2.4C

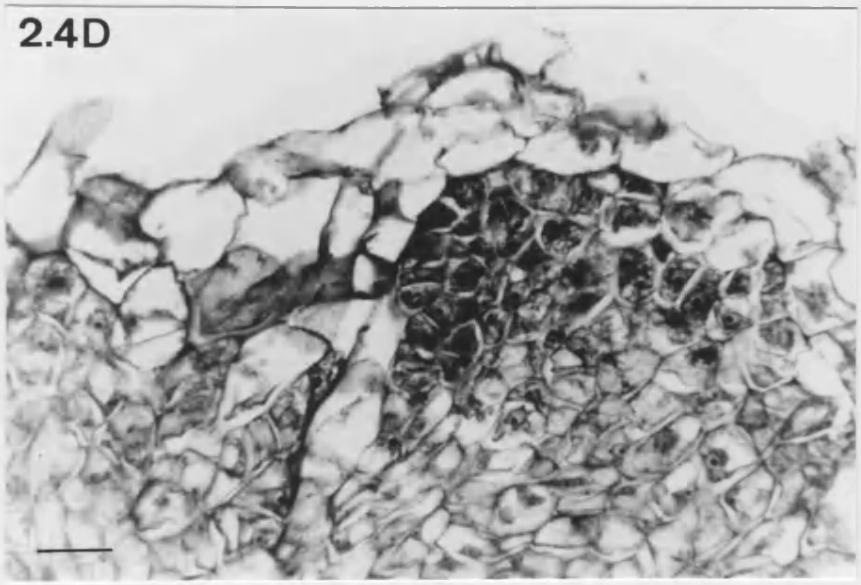

2.4D

Plate 2.4D illustrates the youngest stage at which any definitive meristem was seen. In contrast to the meristematic cells present at the callus surface prior to subculture, these are arranged in an ordered manner with the first indications of a single layered tunica being formed. The whole structure was very small, only five sections, or about 30 μm thick.

The synchronous, multi-regenerative nature of the response described in Chapter 1 Section 4.1 is demonstrated in Plate 2.4E. Eight, or more separate regenerative events are visible, and it appears that all of the meristematic tissue present at the callus surface before subculture has taken part in the formation of the new meristems as few "loose" meristematic cells are visible.

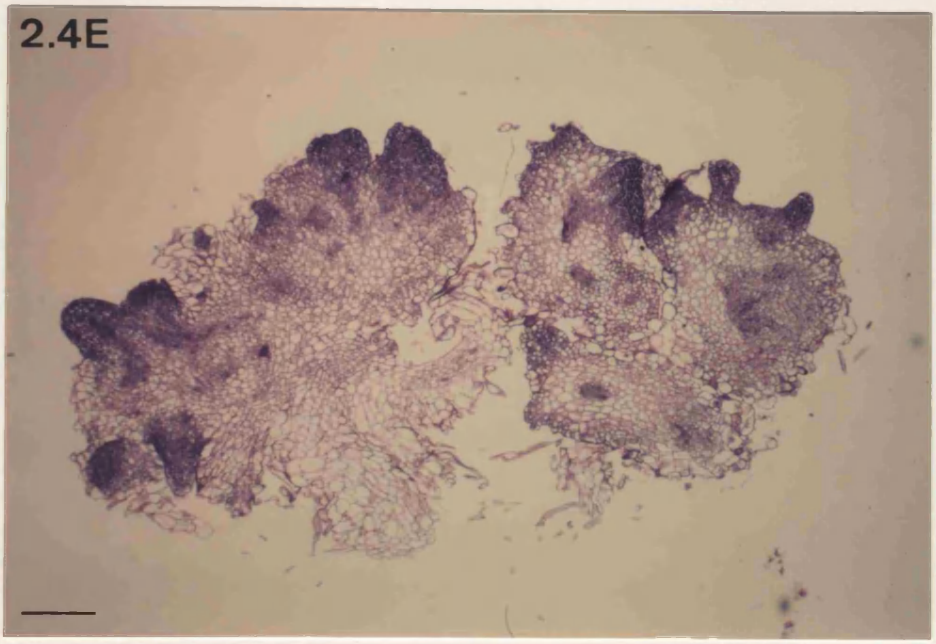
Not all the meristems appear as well formed apices, but this is due to their orientation in many different directions. The section shown here cuts obliquely through some, tangentially through others and only occasionally, and by chance, would a good longitudinal section be obtained. In viewing consecutive sections along a ribbon, however, it was possible to confirm that these are all young shoot meristems. The isolated regions of meristematic tissue seen submerged in the callus tissue in Plate 2.4E are due to transverse cuts through such meristems.

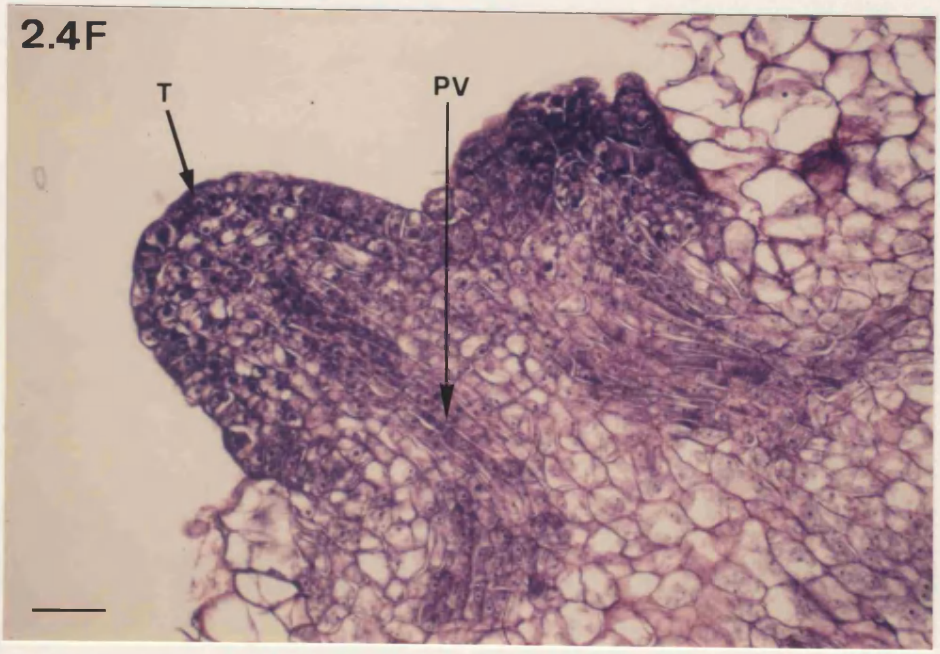
In Plate 2.4F one of the apices is shown in more detail. This good longitudinal section reveals a one-layered tunica, a corpus region and procambium developing between it and the mother callus. The meristem itself appears slightly square possibly indicating development towards the leaf-butress stage. Examination

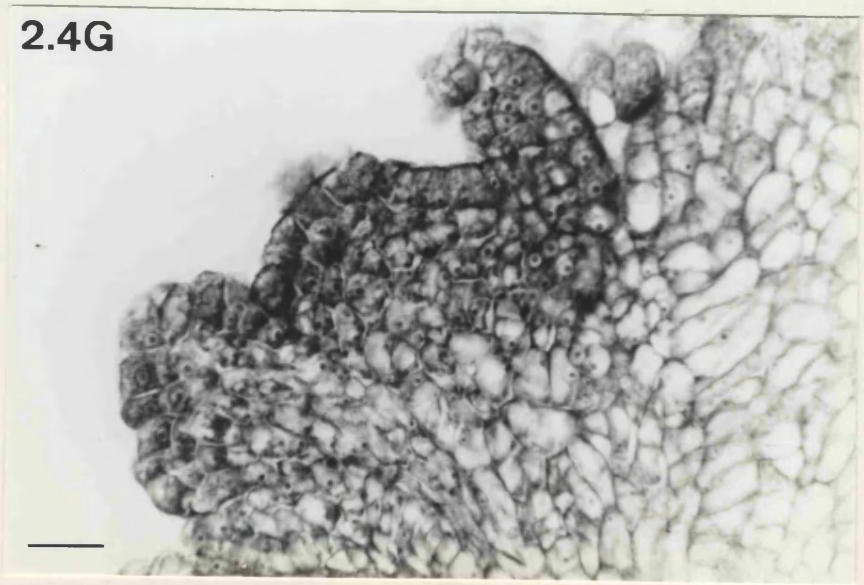
Plate 2.4E Section through a regenerating callus four days after subculture to the shoot induction medium. Approximately eight separate caulogenic events can be seen.
Scale bar = 500μm

Plate 2.4F Detail of an apical meristem from Plate 2.4E showing the single-layered tunica (T) and the development of procambium connections (PV) with the mother tissue.
Scale bar = 100μm

Plate 2.4G Section from different caulogenic tissue showing a young meristem with distinct tunica and leaf primordia.
Scale bar = 65μm


2.4E


2.4F
T
PV


2.4G

of consecutive sections confirmed that this was an independant shoot apex and the smaller meristematic structure to the right to be another regenerating apical region sectioned tangenetially and slightly obliquely.

Plate 2.4G shows a young shoot meristem from another specimen at the same age in which the leaf primordia are clearly visible either side of the shoot apex.

2.4.3 Anatomy and Development of Callus from the Recalcitrant Genotype Coimbra

Of the nine genotypes investigated Coimbra had the lowest caulogenic potential (see Chapter 1, Figure 10). This genotype was therefore examined to identify any possible differences between it and Enkeim.

Coimbra developed to the same pattern as all the other genotypes forming a bi-lobed structure with two enlarging callus ends joined by a region of narrower non-callused tissue. The central section did, however, mature faster than in Enkeim so that after six days a distinctive layer of vacuolated cells was present between the meristematic cells and the eidermal-like layer. This was not seen in Enkeim until eight days.

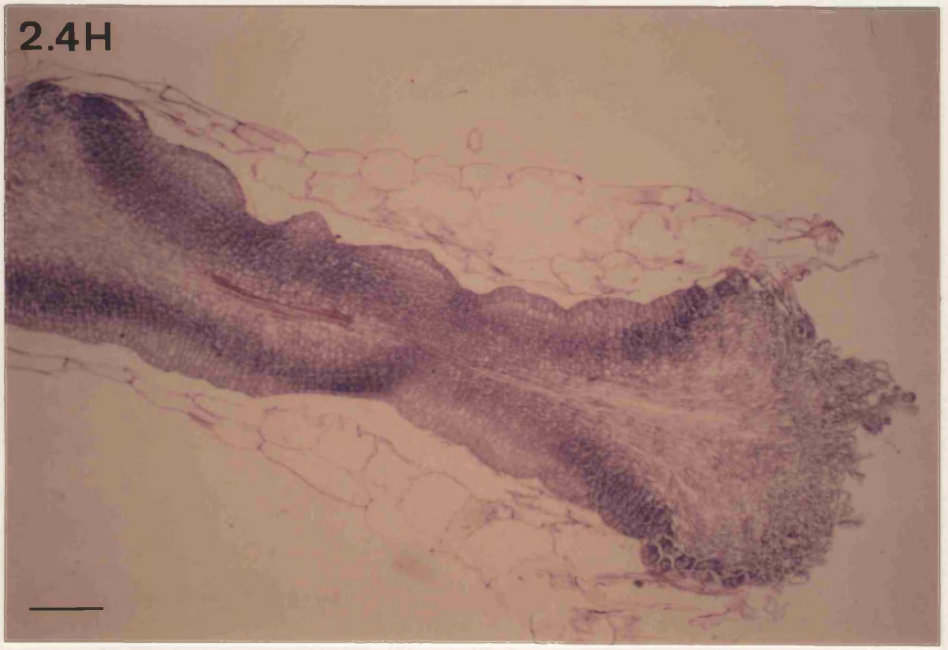
Plate 2.4H demonstrates the vacuolated nature of the cells of the central region and also shows the major difference between the two genotypes. Coimbra has no meristematic cells at the surface of its callus, and this is illustrated from day six to day ten in Plates 2.4H to 2.4J respectively. These show that the callus surface in this genotype consists of a very disorganised

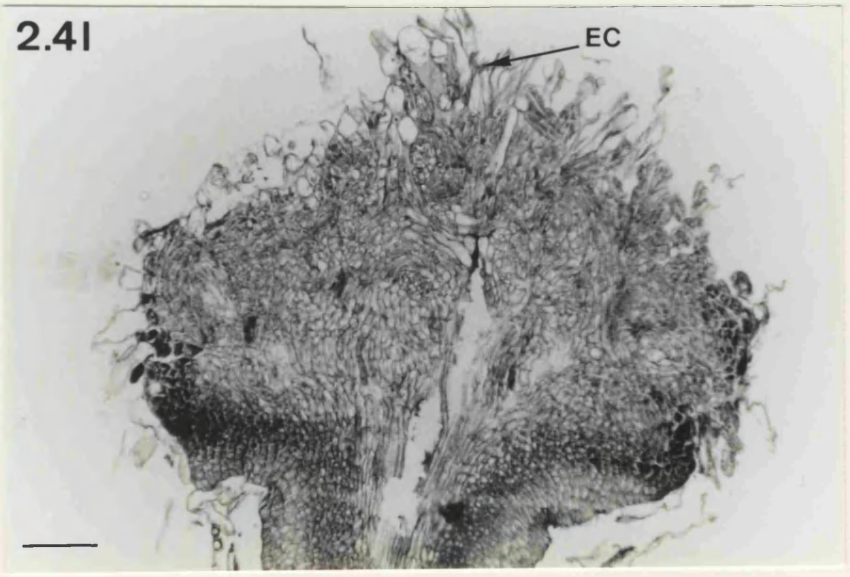
Plates 2.4H - K Development and Characteristics of Callus Tissue from the Recalcitrant Genotype Coimbra

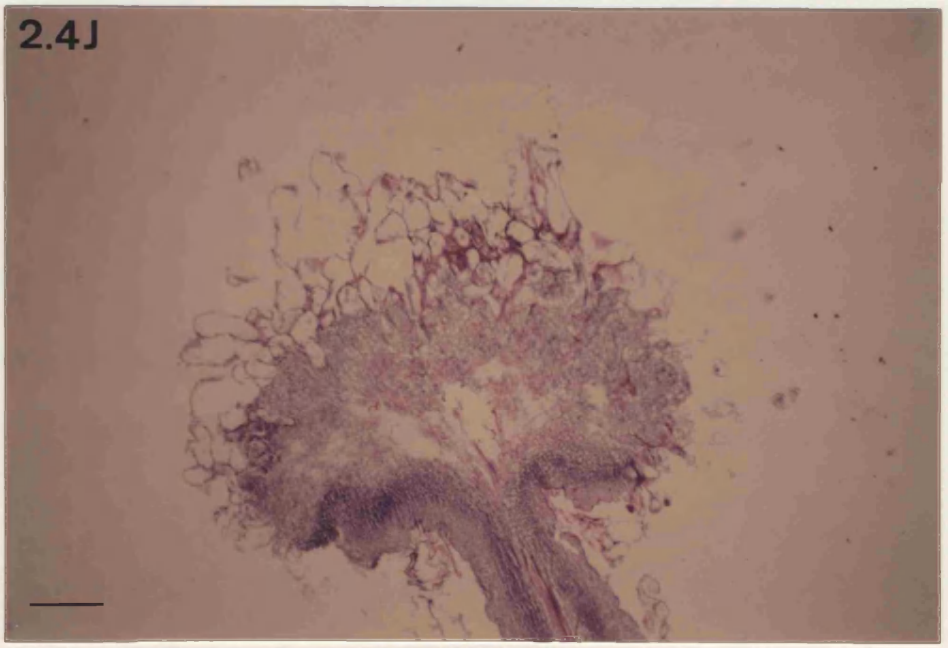
Plate 2.4H L.S. of tissue after six days in the callus induction medium showing the central region and the developing callus. The former possesses a distinct epidermal-like layer below which is a region of more vacuolated cells with meristematic cells in the centre. The callus tissue at each end does not possess a surface layer of meristematic cells as seen in the caulogenically competent genotype Enkeim (see Plates 2.4A and B).
Scale bar = 200μm

Plate 2.4I Section through an eight day old specimen showing the highly disorganised and non-meristematic state of the callus surface and the presence of a large amount of elongated cells (EC).
Scale bar = 250μm

Plate 2.4J Callus tissue after 10 days showing the remnants of many very large thin walled cells projecting from the callus surface. No meristematic cells or regions of meristematic tissue are visible in this section.
Scale bar = 500μm


2.4H


2.4I
EC


2.4J

mass of loosely associated cells. At day six these resemble the non-meristematic cells seen on parts of the surface of specimens of Enkeim, especially in older material, but as the Coimbra explant ages its callus surface becomes increasingly disorganised to the point of being broken up. Plate 2.4I shows many large elongated cells projecting from the surface at day eight. By day ten this phenomenon had increased markedly to produce the structure seen in Plate 2.4J by which time the remnants of what must have been many, very large, thin walled cells are visible at the surface of the callus. Their poor preservation is characteristic of delicate highly vacuolated cells such as those of the hypocotyl cortex described earlier.

Calluses of Coimbra were subcultured onto the shoot induction medium, fixed after various times and sectioned, in the hope of observing a regenerating shoot. However, of the numerous examples examined no caulogenesis was seen.

2.5 The Structure of the Maturing Callus and the Formation and Subsequent Loss of the "Potentially Embryogenic" Tissue

The callus tissue was examined from 14 days after explanting onwards in order to identify the changes that occurred with time and the factors involved in the loss of caulogenic

competence (see Chapter 1, Figure 9) and the formation, and transient nature, of the tissue described in Chapter 1 as potentially embryogenic (PE).

2.5.1 Structure of the Developing Callus

Changes in the structure and distribution of the callus tissue at the eight day stage was described in Section 2.4. After 10 to 12 days exposure to the first stage medium the meristematic cells at the surface of the callus had started to divide and produce steadily larger and thicker layers of darkly stained tissue. In these regions the cells were tightly packed together in contrast to their loose and disorganised nature in the six and eight day stages (see Plates 2.4A and 2.4B). Plate 2.5A shows regions of such cells from tissue 14 days after explanting.

With continuing time in the first stage these meristematic regions increased to become many cells deep, and spread laterally to form bands of meristematic tissue just below the tissue surface, and often removed from it, by a layer of vacuolated cells 1-3 cells thick.

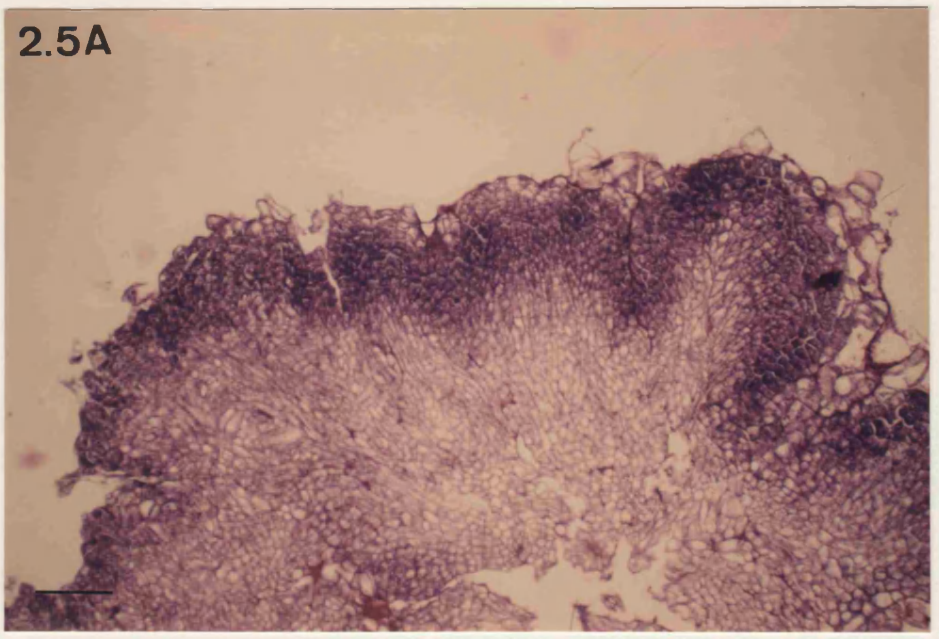
In some cases the meristematic region would develop as an isolated structure, separated from others by areas of disorganised non-meristematic cells. The ratio of meristematic to non-meristematic areas in the callus varied between individuals. Such meristematic regions were rarely seen in Coimbra.

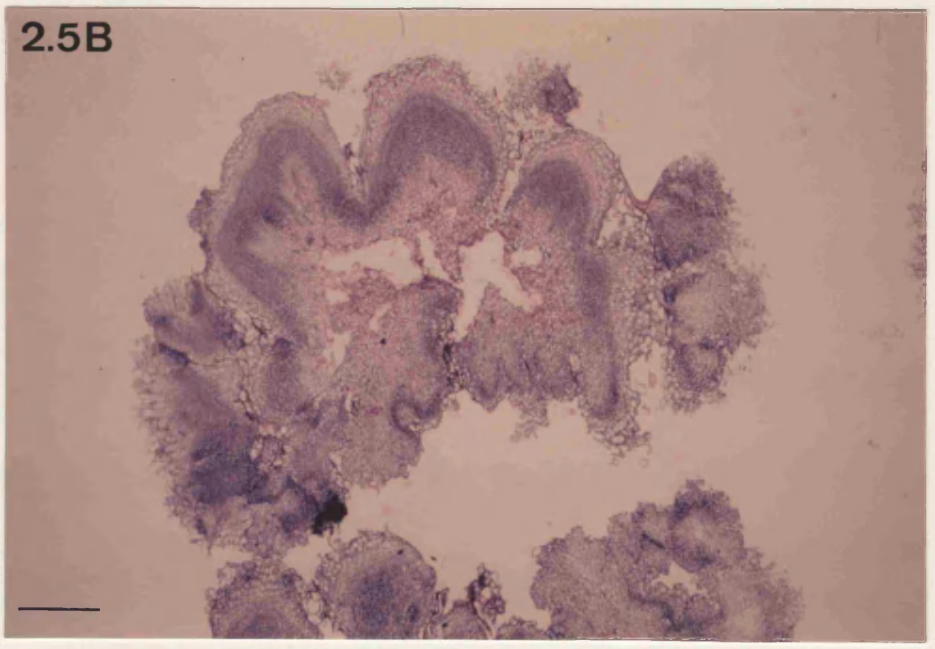
Plates 2.5A - C Structural Changes in the Maturing Callus

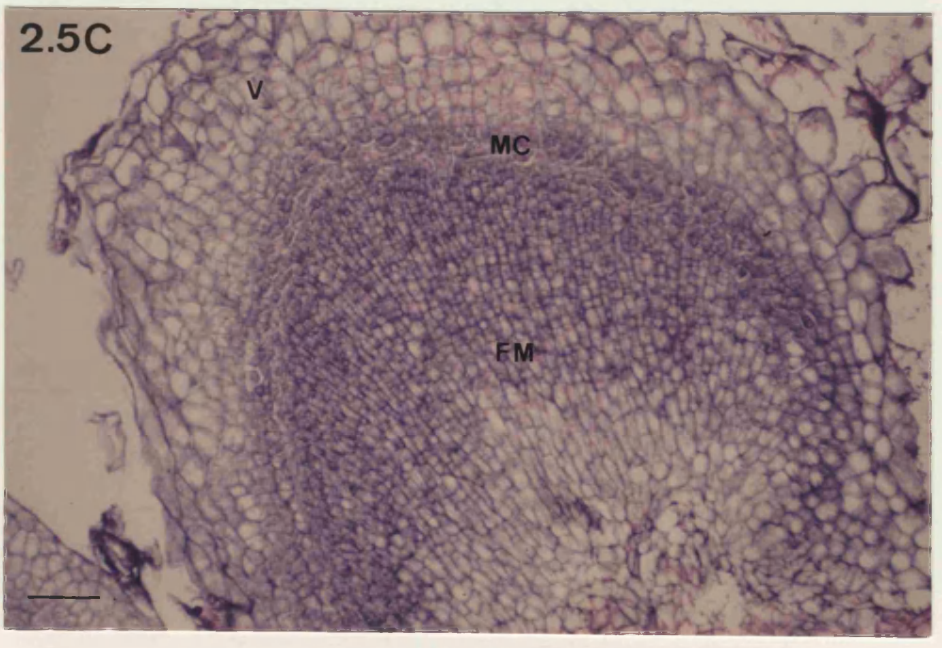
Plate 2.5A The surface of the callus of Enkeim after 14 days exposure to the first stage medium. The meristematic tissue occupies most of the surface area and consists of many layers of meristematic cells packed in a compact and ordered manner.
Scale bar = 200μm

Plate 2.5B Tissue after 20 days in the callus induction medium showing the development of the lobed, or domed, structures.
Scale bar = 500μm

Plate 2.5C Detail of a lobe illustrating their highly structured nature. Three cell regions are visible. Outermost are numerous layers of vacuolated cells (V). This is bounded internally by a narrow region of meristematic cells (MC) which are arranged in a line parallel to the structure's surface. Below this is the file meristem (FM) in which the cells are packed together to form files radiating from the centre of the structure.
Scale bar = 100μm


2.5A


2.5B


2.5C
V
MC
FM

The final form of these meristematic structures is shown in detail in Plates 2.5B and 2.5C. Such structures were common in tissues from 16 days onwards and on closer inspection can be seen to consist of three distinct regions:

1. The outermost region consisted of several layers of large vacuolated cells, which are very lightly stained. These seem to form a well organised surface with the broken edges seen in some sections probably being due to damaged caused by the fixation procedure.

2. Beneath this region there is a thin layer, one to two cells across, of small darkly stained cells. Periclinal divisions have caused these cells to be rectangular in section with the longitudinal edge parallel to the tissue surface. As a unit they form a distinctive line of darkly stained material that is removed from, but parallel to, the surface of the structure seen in Plate 2.5B.

3. The third region is found below the meristematic layer and it consists of many files of cells, resembling a ground, or file, meristem. These cells are partially vacuolated, with visible nuclei and a reasonable degree of staining. They are packed together in an extremely compact and ordered manner to produce many files of almost square (in section) cells extending away from the meristematic layer and into the callus tissue. As the distance from the meristematic layer increases these cells become more elongated with less staining and increasing

vacuolation. The depth of the file meristems varied but they were continuous with the parenchymatous cells of the callus tissue below.

2.5.2 The Presence, Structure, and Subsequent Loss of the PE Tissue

In the genotype Columbia, and to a lesser extent Enkeim and Estland, a smooth undulating surface developed on a proportion of the replicates after culture for 16 days on the callus induction medium (see Chapter 1; Plate 1). This tissue was present for a limited period only, usually four to five days, before it became roughened and sometimes wet in appearance. A number of such specimens from Columbia were fixed and sectioned in order to examine the histology of this tissue and to investigate the transiency of its expression.

Plate 2.5D shows a region of PE tissue from an 18 day old specimen in which a continuous layer of meristematic tissue covers a large proportion of the callus. In this section the smooth, undulating features of the surface that impart the appearance of possibly embryogenic tissue are apparent.

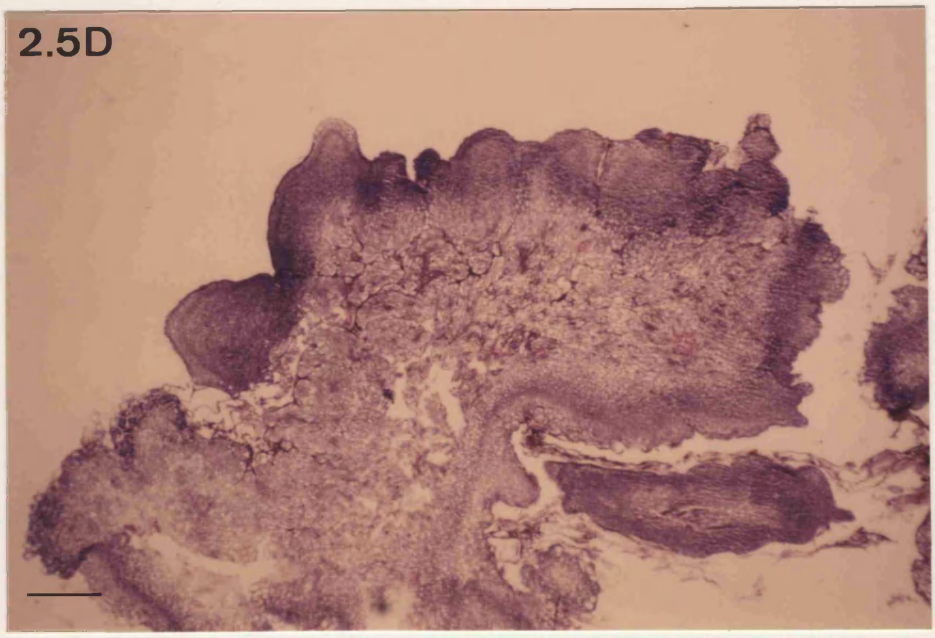
The details of this surface are shown in Plate 2.5E. Here the meristematic cells are present at the periphery of the tissue, in contrast to the those seen in the domed structures described in Section 2.5.1, which were separated from it by layers of vacuolated cells. In this case the outer cells are meristematic

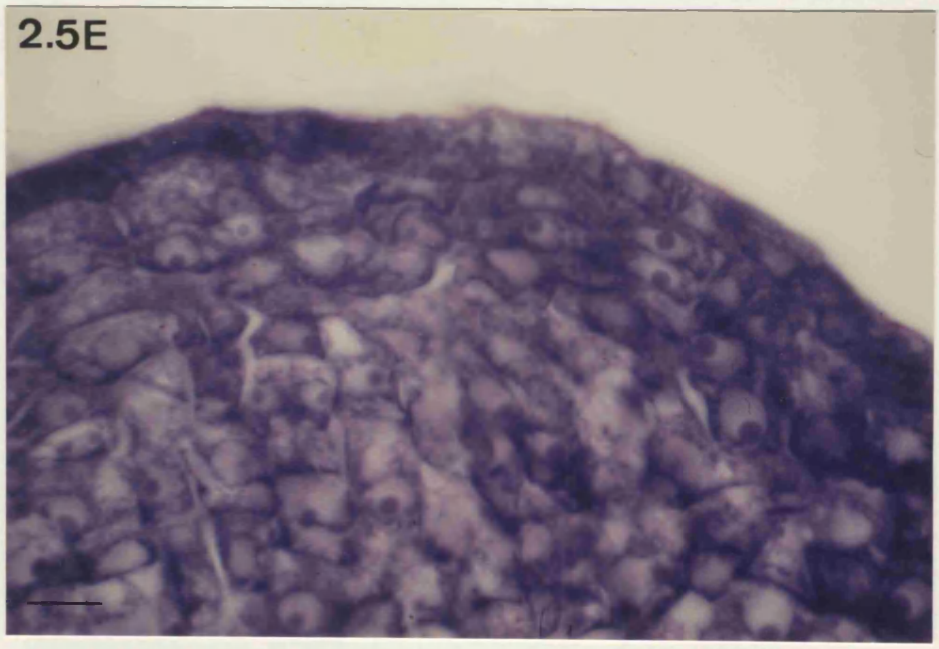
Plates 2.5D - F Presence, Structure and Loss of PE Tissue after 18 - 20 Days in the Callus Induction Medium

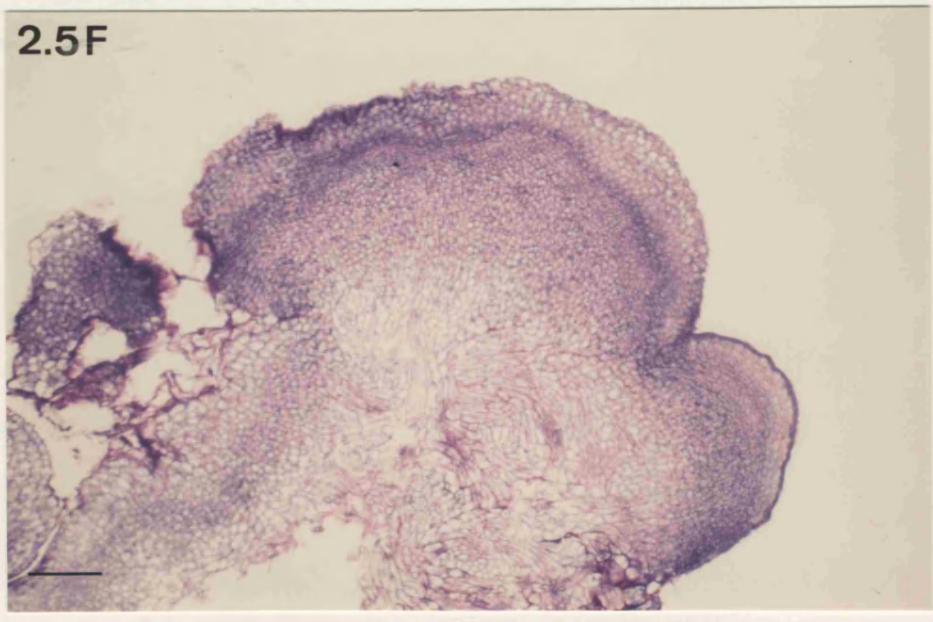
Plate 2.5D Section through tissue of the genotype Columbia identified macroscopically as potentially embryogenic (PE), showing the presence of large areas of meristematic tissue at the surface of the callus. Unlike Plate 2.5B much of this is present at the extreme periphery of the callus and is not separated from it by numerous layers of vacuolated cells.
Scale bar = 500μm

Plate 2.5E Detail of a PE area to illustrate the presence of meristematic cells at the edge of the tissue. There is a distinct outer layer about four cells thick which have their longitudinal axes parallel to the surface.
Scale bar = 20μm

Plate 2.5F A region from the same specimen in which the PE appearance has been lost. In this case the meristematic cells are not present at the surface to impart a smooth texture but are removed from it by a layer, many cells thick, of lighter-staining vacuolated cells.
Scale bar = 200μm


2.5D


2.5E


2.5F

with the peripheral four layers orientated with their longitudinal axis parallel to the surface. These cells must therefore be the result of mostly periclinal divisions.

An alternative surface feature, and one which was often seen in conjunction with that described above, (see Plate 2.5D) is illustrated in Plate 2.5F. This resembles more closely the three layered, domed structures (Plates 2.5B and 2.5C) as, working inwards, there is a region of faintly coloured vacuolated cells, a narrow band of darkly stained tissue parallel to the structure's surface and finally a mass of partially stained cells which eventually merge into the parenchyatous tissue. In this case, however, the cells of the third region do not appear to be arranged in regular files.

2.6 Presence of Embryoid Structures in Callus Tissue of Enkeim

Whilst viewing a number of sections from tissue exposed to the callus induction medium for 20 days, the two embryoid structures shown in Plates 2.6A and 2.6B were seen in a specimen from the genotype Enkeim. This was the only occasion that such structures were observed.

The broken nature of the background tissue is confusing, but from Plate 2.6A it appears that the meristematic structures shown are submerged in the callus and are possibly developing with their apical ends inwards. This, however, is by no means clear. Each embryoid has a suspensor-like attachment to the meristematic

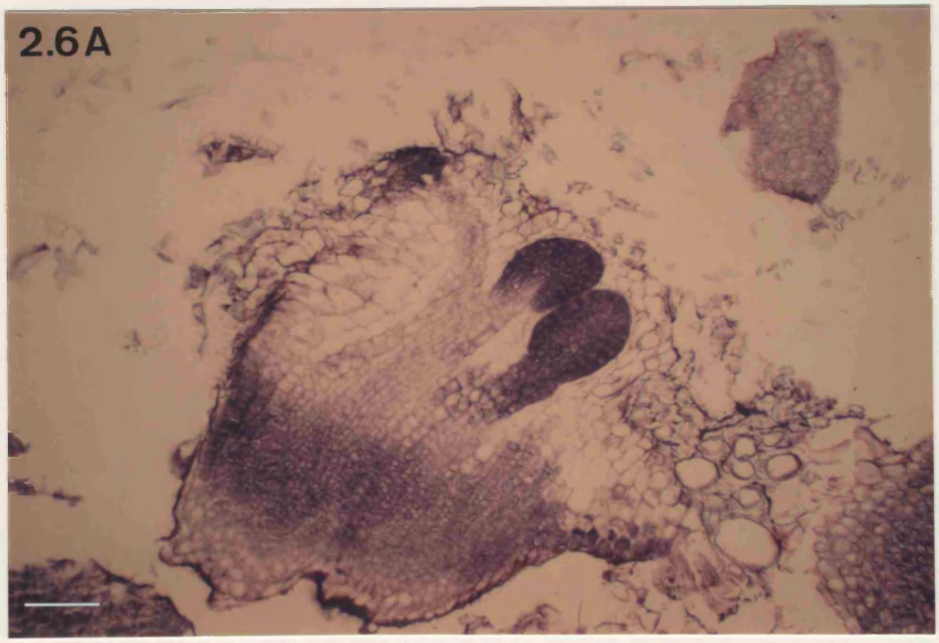
Plate 2.6A - B Presence of Two Globular Structures in 20 Day Old Tissue of Enkeim

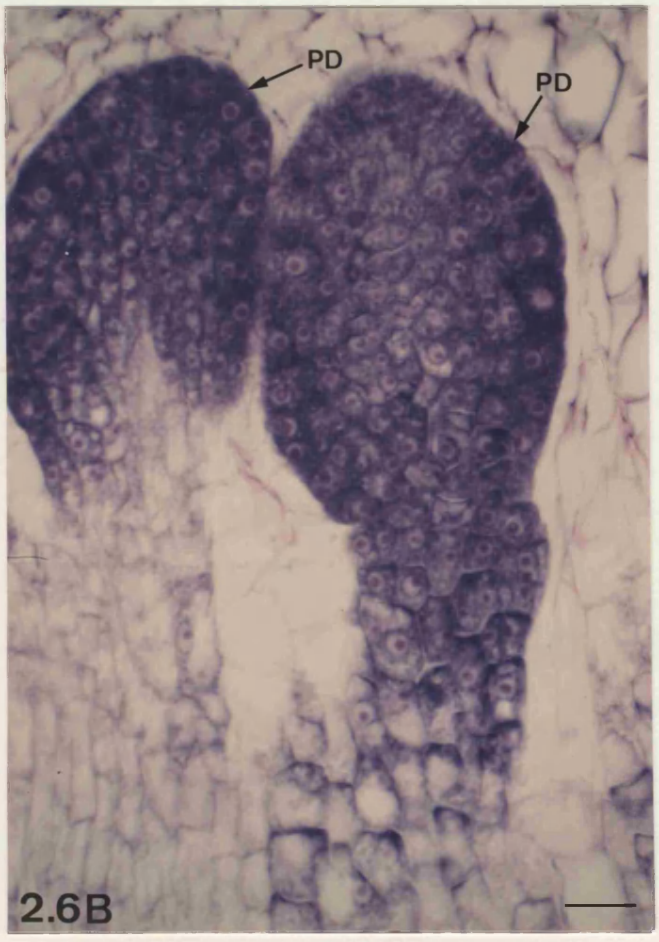
Plate 2.6A Two globular embryoid structures developing in tissue of Enkeim. Each has a suspensor-like attachement to the file meristematic region below. It appears that these structures are submerged within the callus but from the broken nature of the tissue in this section it is possible to discern the direction of the callus surface.

Scale bar = 200μm

Plate 2.6B The globular structures under higher magnification showing the suspensor-like structures to be four to five cells wide and the presence of a highly meristematic protoderm (PD) around the embryoids.

Scale bar = 50μm


2.6A


PD
PD
2.6B

region below indicating that it may have originated from this tissue. The greater detail afforded in Plate 2.6B shows the suspensor-like structures to be multi-cellular, four to five cells thick, and the globular heads to be highly meristematic possessing a distinct protoderm.

2.7 Shoot Regeneration from Calluses of Chisdra after 16 - 18 Days Exposure to the First Stage Medium

The genotypes Estland and Chisdra were distinctive in their ability to regenerate shoots at frequencies of 70-80% after 16 - 18 days culture on the callus induction medium. This contrasted with the other genotypes which had completely, or almost completely, lost their caulogenic potential by this time; see Chapter 1, Figure 9.2.

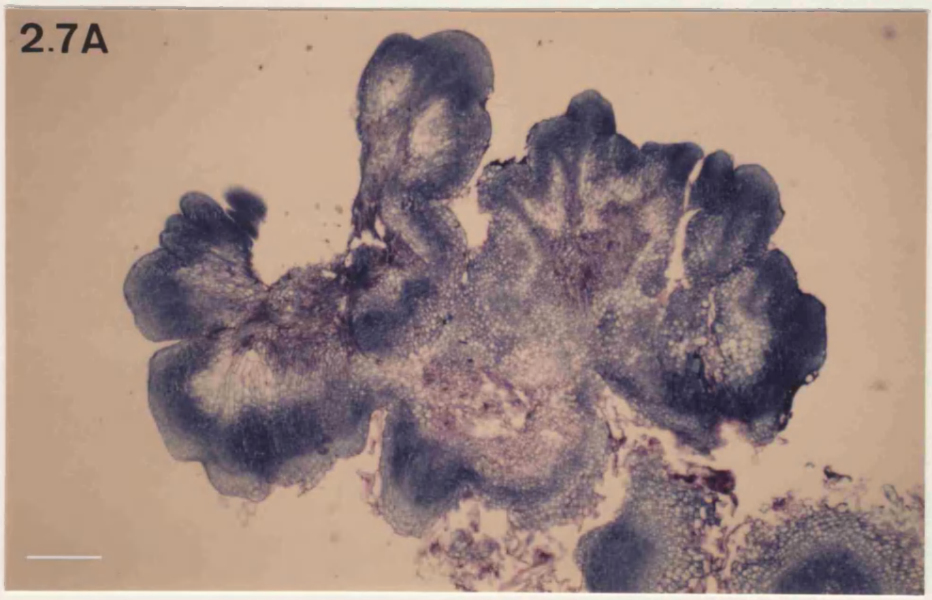
Calluses from the genotype Chisdra were cultured for 18 days on the first stage medium, fixed, and sectioned in order to study their histology, and investigate the reasons for the prolonged competence of this tissue. Plates 2.7A and 2.7B illustrate the state of the tissue at this time.

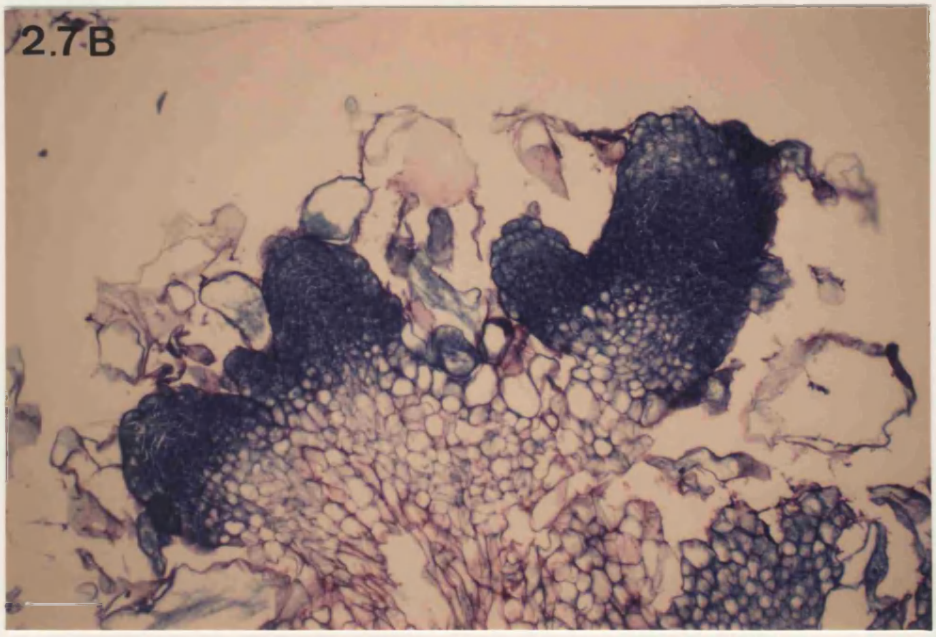
No major differences were seen between this tissue and that of Enkeim or Columbia after an equivalent time in the first stage; Plate 2.7A confirms this. As in Enkeim the callus consisted of a mixture of non-meristematic cells and meristematic cells organised into large, heavily staining regions. Often this consisted of the three regions described in Section 2.5 including

Plates 2.7A – B Structure of the Callus in the Genotype Chisdra after 18 Days Culture on the Callus Induction Medium

Plate 2.7A Tissue in the callus region showing extensive development of meristematic tissue. As in the genotype Enkeim, at this stage, these consist of many cells layers, with close cell-to-cell contact and compact organisation.
Scale bar = 500μm

Plate 2.7B Detail of a number of small, isolated, organised meristematic structures. Such regions were commonly seen in tissue of this age in Chisdra but not in Enkeim.
Scale bar = 200μm


2.7A


2.7B

layers of vacuolated cells at their periphery. No loose meristematic cells, characteristic of caulogenically competent tissue in Enkeim, were seen at the callus surface of the specimens of Chisdra examined.

The only distinctive feature observed were the structures illustrated in Plate 2.7B. These isolated, organised meristematic regions were smaller than any seen on the other genotypes at the equivalent stage. They were, however, quite common on Chisdra. Whether the shoots produced by this genotype during its extended period of caulogenic competence were derived from these structures, is not known as tissue from this genotype at this age was not sectioned after subculture to the second stage medium. Indeed, the large size and poor synchrony of the caulogenic response of such tissue would have made this exercise problematic.

2.9 The Presence and Distribution of Starch Grains in Subcultured and Non-Subcultured Tissues

Whilst examining the distribution and composition of the xylem tissue by phase-contrast microscopy numerous small rounded particles were seen in some cells; viewed with polarised light, these structures were highly bifringent and they showed the characteristic Maltese-cross shape, identifying them as starch grains. Subsequent investigation found starch to be present in this form in a number of specific tissue types and at different stages of the culture system.

Plates 2.8A - C Distribution of Starch Grains at Various Developmental Stages from Cultured Tissues of Enkeim and Coimbra Viewed with Phase-Contrast Microscopy

Plate 2.8A The central region of Enkeim six days after explanting. In the genotype there is no vacuolation of the surface cells and no starch deposition.

Note the presence of the xylem elements along the length of the central region.

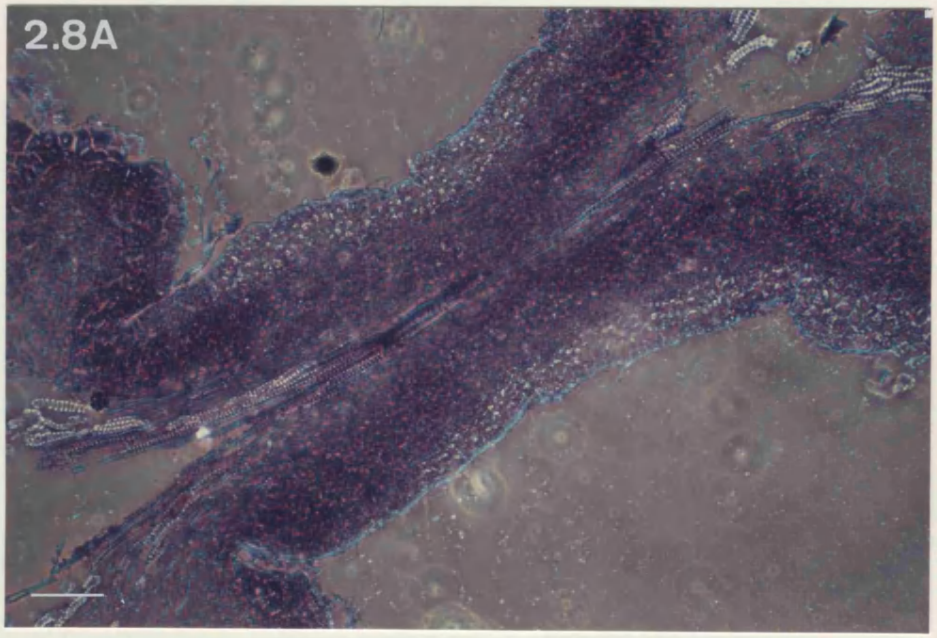
Scale bar = 200μm

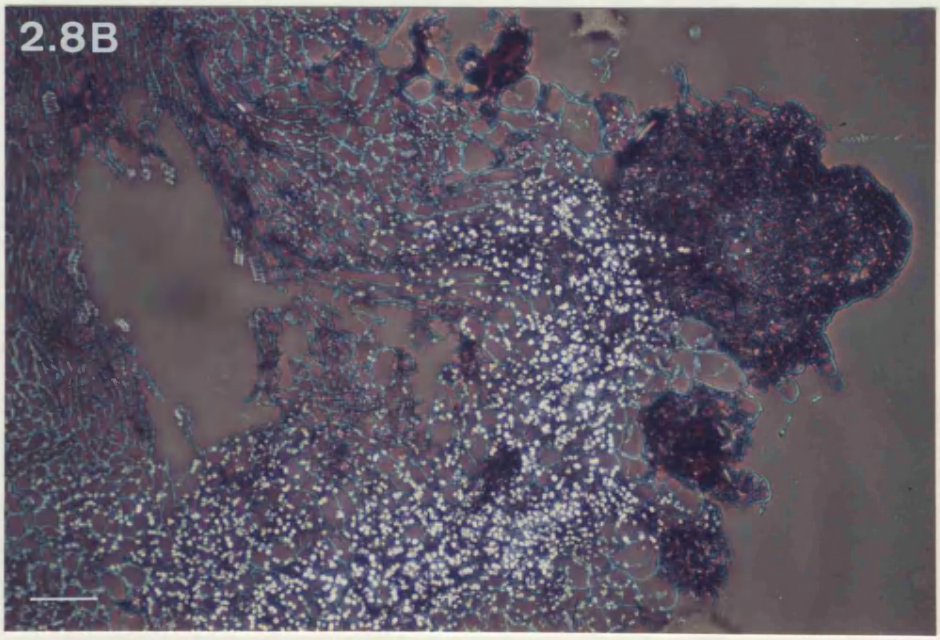
Plate 2.8B Large scale deposits of starch seen in the parenchymatous cells of the callus regions 4 days after subculture to the second stage medium. No starch is seen in the meristematic cells of the regenerating shoot.

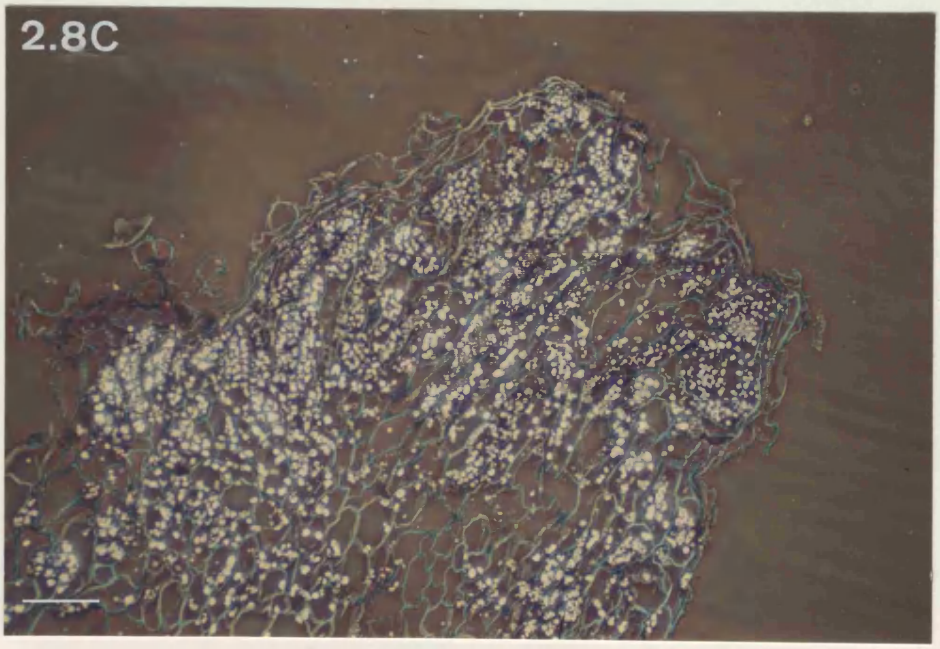
Scale bar = 200μm

Plate 2.8C Massive deposition of starch in the large, vacuolated cells of non-caulogenic tissue from the genotype Enkeim 8 days after sub-culture.

Scale bar = 200μm


2.8A


2.8B


2.8C

2.8.1 Distribution in the Central Region

Starch grains were never seen in tissues before the six day stage. By this time the more developmentally advanced genotype Coimbra showed considerable deposits which were restricted to the epidermal-like layer, and especially the vacuolated cells below it. None were visible in the meristematic tissue unless viewed under very high magnification, and then the grains were very small, few in number and scattered sparsely amongst, and within, these cells. In contrast the vacuolated cells contained much larger particles at between four and ten per cell.

Plate 2.8A shows a similar pattern of starch deposition as described above, but present in tissue from Enkeim after ten days in the callus induction medium. The particles are clearly restricted to the more vacuolated cells at the edge of this tissue.

2.8.3 Distribution in the Callus Tissues

Starch grains were not seen in the parenchymatous or meristematic cells of the callus in the first stage medium, regardless of the time in culture or the genotype. Only in vacuolated cells produced exterior to the organised meristematic tissues (14 - 20 days) were any visible. These large vacuolated cells contained numerous starch grains of a size and distribution similar to that seen in the vacuolated cells at the periphery of the central region.

In contrast to the situation with tissue cultured continuously on the callus-induction medium, starch grains were widespread in the parenchyma, but not the meristematic cells, of

subcultured material (see Plate 2.8B). This was observed in tissues from Enkeim and Coimbra and at times the deposition was so heavy that it obscured the cellular details. The grains were seen as a mass of bright particles in the vacuolated cells of the callus.

The heaviest deposits observed were seen in tissue from the few specimens of Enkeim which failed to regenerate shoots. These were sectioned in order to determine the differences between them and the majority from this genotype, which were highly caulogenic. Plate 2.8C shows the presence of very heavy deposits in such tissue ten days after subculture to the second stage medium.

DISCUSSION

DISCUSSION

Tran Than Van (1981a) outlined the qualities required of a culture system if it is to act as an effective model for the study of the fundamental aspects of plant morphogenesis. She stresses the need to utilise simple systems with simplified media and the ability to compare recalcitrant and responsive tissues which differ in genetic complement as little as possible; for example different genotypes of the same species.

The organogenic system developed and described in Chapter 1 satisfies these criteria allowing the second part of the current study to exploit these qualities and examine the pattern and distribution of cell types associated with increasing exposure to the first stage medium, and the onset of organisation induced by subculture to the second stage. It was hoped that such a histological approach would provide a better understanding of the structural aspects of organogenesis in A. thaliana, and indeed it is considered that this has, at least partly, been achieved, with the result that a number of questions generated by the manipulative studies of Chapter 1 have been addressed. These provide a better understanding of the structural factors behind the acquisition and loss of competence and determination for different organogenic fates, the reasons for the recalictrant nature of the genotype Coimbra and an appreciation of the lack of somatic embryogenesis in tissues of Columbia.

Early studies centred on the development of the tissue from day 0 to 20 on the first stage medium in order to examine the histological changes that occurred in the tissue, as the time to subculture was increased. This allowed the structure of the tissue to be correlated with the various organogenic states identified in Chapter 1, Sections 4 and 5.

As discussed in Section 2.2 of this Chapter explanting hypocotyls onto the first stage medium led to the development, and subsequent loss, of dumb-bell-shaped specimens (see Plates 2.2A to 2.2H) to form relatively large pieces of callus. Longitudinal sections through tissue soon after explanting revealed that very little cell division occurred during the first days exposure to the first stage medium. The first visible changes involved the appearance on either side of the xylem elements of deeply stained cells with large nuclei, in the region where one would expect to find the cambium, primary phloem and pericyclic tissues (see Plate 2.3A).

Sections through progressively older cultures showed that the tissue in the central and terminal regions of the bi-lobed structure developed differently. The central region, from which the majority of root regeneration took place, was derived from extensive divisions of the meristematic cells seen in Plate 2.3B and, apart from the vascular strand, consisted almost entirely of these small isodiametric cells (see Plates 2.3C and 2.3D). The average cell size was dramatically reduced between the two and four day stages and although some increase in girth was observed during this period it did not approach or reflect the degree of cell

division. Whether this represents the Division Phase (see Introduction, Yeoman, 1970) for this tissue is not clear as it must be stated that discriminating between this stage and that of a Differentiation Phase was impossible as the specimen grew throughout the culture period (see Chapter 1, Figure 8). It is possible that the beginning of the Differentiation Phase had not been reached by the end of the 20 day observation period and it therefore seems impractical to apply these terms to the system as it existed here.

The most striking feature of the central region was the extremely ordered manner in which the cells were packed together (see Plates 2.3C and 2.3D). The cell divisions must have been under some strong controlling factor as they occured, after the first few days, mostly in the periclinal direction. No oblique divisions took place, no intercellular spaces were seen and a highly organised tissue was produced in this region, which formed a smooth, if undulating, surface.

In Enkeim a epidermal-like layer was seen around the central region from six days onwards (see Plates 2.3I and 2.3J). This was distinctive from the meristematic cells below it by its greater vacuolation and smaller nuclei. Before this time ie. at day four the meristematic cells were outermost forming what would appear to be a protodermal layer to this region (see Plates 2.3C and 2.3D). This corresponds to the time of greatest rhizogenic competence.

Development of the callus tissue at the terminal regions differed from the above in that its origin was less clear, it consisted of numerous cell types and grew in a less controlled or organised manner. From the sections obtained it was not possible to determine the exact origin of this tissue but it was possibly derived from less visibly meristematic cells at each cut end of the explant, perhaps the exposed procambium or pericyclic tissue.

The bulk of the callus regions consisted of vacuolated parenchymatous cells which appeared to have no regular division pattern but divided in all directions producing a disorganised tissue with some intercellular spaces. Also present in these regions were a number of elongated cells as described in Section 2.1, which, although spread throughout the callus, were often present just below the surface. They appeared to be continuous with similar cell types seen in the central region which formed a sheath around the xylem elements (see Plates 2.1C, 2.1D and 2.3J).

The xylem also extended out from the central region into the callus (see Plate 2.4A and 2.11C), although this was not seen until after eight days. It was not possible to determine whether this was in the form of a continous system of elements spreading developing as a result of differentiation-dependant pattern formation (Sachs, 1978). Some tracheids were seen in this region suggesting that isolated cytodifferentiation was also occurring.

The arrangement of meristematic cells in this callus also differs from that in the central region. Unlike the latter, in which these cells constitute the major part of the tissue, the meristematic cells were restricted to the periphery of the callus.

Plate 2.3A shows that few meristematic cells were present in the small amount of callus formed by the fourth day, but that very considerably multiplication had taken place by the six and eight-day stages, by which time the majority of the callus surface was covered by a thin disorganised layer of these cells (see Plate 2.4A and 2.4B).

The origin of these meristematic cells is not known but it is possible that they arose from a few isolated cells present at the earliest stages of the callus development or from the cambian/pericyclic region around the xylem. The latter would mean a common origin for the meristematic cells of the central and callus regions. Alternatively, the parenchymatous cells of the callus could have been induced to divide by the first stage medium and produce the meristematic layer characteristic of the six- and eight-day stages.

Information gathered from observations of the development of the specimens in culture, and especially the distribution and structure of the meristematic cells at the time of subculture, has allowed a possible explanation for the pattern of organogenic competence described in Chapter 1 to be developed.

The histological work has shown that there is a definite correlation between the presence and structure of these meristematic cells and the ability to regenerate. When none, or very few were present, as in tissue exposed for only two days on the first stage medium, the tissue was competent for neither root nor shoot formation. By day four the specimen was competent for root but not shoot production and this correlates with the presence

of meristematic cells as a distinct sheath around the xylem elements and their almost complete absence from the small amount of callus formed at each end by the this time (see Plate 2.2A). High frequency caulogenesis took place on the second stage medium after first-stage periods of between six and ten days and this corresponds with the presence of the thin layer of loose disorganised meristematic cells present at the periphery of the callus at this time (see Plate 2.2C and 2.4A).

Callus which lacked meristematic cells in this region was not capable of forming shoots on exposure to the second stage medium. As stated they were not present on specimens subcultured after two or four days on the first stage, nor were they ever seen in the organised central region; shoots never arose from this tissue. Most striking, however, were the sections obtained form the recalcitrant genotype Coimbra (see Plates 2.4H - 2.4K). These showed that its callus of all ages, despite good cell division and growth, did not possess a peripheral layer of meristematic cells. The lack of meristematic cells in this area seemed to be correlated in this genotype with its very poor caulogenic competence.

More subtle was the effect of continued exposure to the first stage medium. Plates 2.2E to 2.2H show how, from day twelve onwards, the form in which the meristematic cells were present changed. Instead of being arranged in a thin disorganised layer, they had undergone numerous periclinal divisions to form bands, or discreet regions, of meristematic tissue many layers deep (see Plate 2.5A). The ultimate result of this is the formation of the domed, file-like meristems seen in the tissue exposed to the callus

induction medium for 20 days and illustrated in Plate 2.5C. Closer examination of this structure reveals that it consists of three regions which are the results of periclinal divisions taking place parallel to the tissue surface. Increasing time in the first stage medium beyond approximately ten days is therefore associated with a reduction in the amount of loose meristematic cells, an increase in the orderlines and size of the meristematic regions and a fall in the caulogenic competence of the tissue.

Studies of the first few days after transfer to the second stage medium demonstrates beyond doubt that the regenerating shoots originate from the callus surface and from the loose meristematic cells in particular (see Plates 2.4C to 2.4G). Thus, from this and the evidence presented above, it is postulated that meristematic cells, present in the form of a thin disorganised layer are a requisite for caulogenesis, and tissues which do not possess such cells in this form will not regenerate shoots at significant levels.

With respect to rhizogenesis the situation is less clear. The presence of meristematic cells in the central region is necessary for rhizogenesis but as described in Chapter 1 this competence is lost after four days on the first stage medium in favour of caulogenesis. Why this occurs is not clear but a possible competitive effect, involving the suppression of rhizogenesis by the tissue's increasing commitment to shoot regeneration, was postulated earlier,. Anatomical examination of this tissue, however, reveals some structural changes at this time which might be significant. Plates 2.3I and 2.3J both show the

formation of relatively large vacuolated cells with small nuclei making up the outer two to four rows (depending on the time exposed to the first stage medium) and the presence of a distinctive epidermis-like layer. These cells appear to be derived from and have replaced the highly meristematic cells that formed a protodermal-like surface to this region after four days (see Plate 2.3C).

When viewed under phase-contrast microscopy the vacuolated cells, present at the periphery of the central region from six days onwards, were seen to contain many starch grains (see Plate 2.8A). Such deposits were also found in the vacuolated cells that formed the outer layers of the domed structures in older callus and the parenchymatous cells of the terminal calluses after subculture to the second stage medium (see Plate 2.8B). All of these cell types appeared to be differentiated and were not involved in morphogenesis. The presence of starch in such cells would seem to contradict the findings of Thorpe and Murashige (1970) who suggested that they had a causal role to play in the organogenic process.

The only evidence that such structural developments affect the organogenic competence, however, is that they are correlated temporally with its loss. It is probable that the vacuolated cells have lost the ability to divide and are therefore unable to respond in a coordinated manner with the underlying meristematic cells after an exposure to the inductive signal.

Conversly the cells of the meristematic protodermal layer may well still have the ability to divide in concert with those in the tissue below to form a new root structure.

That meristematic cells must be present before organogenesis can take place is not surprising as it is these cells that will form the meristematic region of any derived organ, but it is apparent that they must also be present in a specific form or structure. The idea that different arrangements of such cells will produce different organs under more or less the same inductive conditions is not a concept commonly encountered in the literature. From these observations it would appear that the tissue posseses what might be called an inherent structural state at the time of introduction to the inductive conditions (ie. the second stage medium) and that this is of great importance in determining the type and quantity of the morphogenic response.

The fact that subculture from the first stage medium is required before organogensis can proceed is fully reported in Chapter 1. Continued exposure to the first stage causes the formation of large, increasingly organised regions of meristematic tissue via mostly periclinal divisions of these cells. In order for organogenesis to take place, however, a shift in the direction of cell division must occur (Green, 1980; Furuya, 1984) onced formed a young meristem will regulate its own cell divisions and development (Green, 1980; Linthilac, 1984) and therefore the critical stage in caulogenesis is the creation of the tunica and corpus (although it is possible that the tunica may be capable of forming the latter).

The formation of these structures was a difficult phenomenon to study, however, because they were either visible, meaning that the process was all but over, or not visible in which case they were impossible to observe. Plates 2.4C and 2.4D show the earliest stages of shoot regeneration observed. Both illustrate the presence of a distinctive tunica demonstrating that anticlinal divisions must take place if such a structure is to be formed and that both anticlinal and periclinal divisions are necessary in the corpus if the shoot bud is to develop to a self-generating meristem. Controlled and continued periclinal divisions are not capable of generating these patterns and it would appear that the cells of the large meristematic regions (the development of which seems to be associated with the loss of caulogenic competence) are locked into this pattern, either physiologically due to prolonged exposure to the first stage medium or due to the physical forces acting within them. Many plant forms are considered to be self-regulating and this is thought by some workers to be due to the physical stresses within them determining the directions in which cells divide (Green, 1980; Linthilac, 1984). Thus the multi-layered meristematic regions present in the older tissue may be restricted to periclinal divisions only and so be unable to respond to the shoot-inductive conditions upon subculture to the second stage.

It is not difficult to envisage that the meristematic cells at the surface of the callus, as seen in Plates 2.4A and 2.4B, have less constraints on them and could be influenced to a greater extent by external factors. Their loose disorganised

patterning may also permit both periclinal and anticlinal divisions to take place with greater ease than in the larger, well ordered meristematic regions.

This leads one to consider the extent to which the non-caulogenic meristematic cells of the older tissues are epigenetically or physiologically different from the competent meristematic cells at the callus surface of 6-10 day specimens, or, indeed, from the rhizogenically-competent cells of the central region. The current study was not designed to address this question but it is possible that the only difference between the meristematic cells of the different regions is their arrangement, and the constraints that this puts upon the division patterns that can take place. The importance of the cell wall and the structural implications that it has for plant development was commented on earlier (see Introduction). Any future study on the differences between the cells of the different meristematic areas would be important as it could address directly the nature of the states of competence and determination in plant systems and whether they are based on the epigenetic qualities of the individual cells or whether they are a consequence of the cells being limited in their morphogenic potential by their position within a given tissue. Such investigations could hopefully ascertain whether this is by positional information in sensu Holder (1979), or purely due to the physical forces acting on them (Lintilhac, 1984).

It can also be seen that the growth regulators have a central role to play in the organogenic process. Continous culture on the first stage medium containing 10^{-6}M 2,4-D and 10^{-7}M BAP

causes meristematic cell production and subsequent multiplication and organisation into that typical of the central region (see Plate 2.3C and 2.3D) or the callus regions (see Plates 2.5A to 2.5C). However, no organogenesis took place on this medium.

Subculture to the second stage medium, which effectively removes the auxin and increases the cytokinin by a factor of ten, induces organogenesis, the type and quantity of which is dependent on the duration of the first stage. It has been shown that rhizogenesis does not require BAP to be present in the second stage (see Figure 13) and therefore it must be the removal of the 2,4-D which is either the inductive or permissive signal. Caulogenesis, on the other hand, requires an exposure of 24-48 hours to 10^{-6}M BAP for effective shoot formation to occur suggesting that this growth regulator acts as an inductive signal for shoot regeneration.

The two organogenic processes are thus different with regards their inductive requirements and histology. In rhizogenesis whole regions of the central section are involved in forming the new organ and there is no question that this is a multicellular event, with the removal of the 2,4-D triggering or permitting a organisational change. This probably involves changes in the direction of cell division and/or cell extension in a pre-determined direction. Indeed, the process is so rapid - only a matter of hours until its first indications - that it must be asked if the organs were not in fact preformed, in other words determined in the first stage, and that the effects of subculture are simply permissive.

The hypocotyl is, in many respects, a root-like organ and it is likely that the meristematic sheath that developed around the vascular strand could be regarded as pericycle. Although much larger and numerous in cell number than the in vivo pericyle the reaction from the central region appeared to have much in common with normal lateral root formation.

Shoot regeneration differed in this respect as it was most certainly not the result of the release of a pre-formed structure on transfer to the second stage medium but involved de novo meristem formation. It is not possible to tell from the sections obtained in this study whether these meristems originated from single cells or small groups working in concert, as described by Williams and Maheswaran (1986), whether such groups were all the daughters of one cell or were derived from different derivitives. As stated, studying the very early stages of the caulogenesic event was not easy as it was difficult to identify the meristems before they were relatively large and thus well-formed. All the regenerants seen had a single layered tunica (see Plates 2.4F and 2.4G) which corresponded with that seen in sections of in vitro examples (results not shown).

The genotype Chisdra may well provide an exception to the above, however, as there is some evidence that this genotype regenerated from pre-formed meristem-like structures if exposed to the first stage medium for long periods. Plates 2.7A shows that there was none of the loose, caulogenic, meristematic cells on the callus surface after 18 days, only large organised regions. Nevertheless tissue of this age was still capable of caulogenesis

at considerable frequencies after subculture to the second stage. The isolated meristematic structures shown in Plate 2.7B were characteristic of this genotype but were not seen in tissues of Enkeim, Columbia or Coimbra. These organised meristematic regions were much smaller than those seen in the other genotypes and if they were acting as preformed shoot meristems this would explain the extended caulogenic competence of this tissue. This is speculation, however, and more detailed examination of this genotype is required if the reasons behind this ability are to be elucidated.

One of the disappointing aspects of the morphogenic studies in Chapter 1 was the failure to achieve somatic embryogenesis from tissues of A. thaliana, especially after tentatively identifying a particular callus as being possibly embryogenic. This tissue was formed on callus cultured for a relatively extended period on the first stage medium (10^{-6}M 2,4-D/10^{-7}M BAP) and it was distinctive in its white colour, smooth and shiny texture and folded shape. Sectioning such material produced by Columbia revealed more information about this tissue type.

A characteristic feature of the smooth tissue was the presence of meristematic cells packed in a highly ordered manner at its surface. This contrasted with the file-like meristems on specimens of the same age seen in tissue from Enkeim (see Plate 2.5C) in which the meristematic cells were separated from the surface by a few layers of large, vacuolated cells. Such file-like meristems, which were a common feature of the tissues at this age,

had a line of highly meristematic cells just below the surface which produced large vacuolated cells to its exterior and the much smaller meristematic cells of the file-like region to its interior. In the case of those tissues identified as PE, and shown in Plates 2.6A and 2.6B, the meristematic cells are seen to be present at the very periphery of the tissue. In Plate 2.5E the cells that form the outer four or five layers are seen to be heavily stained and to have their longitudinal axis parallel to the tissue surface. The very close packing and small size of these cells would be responsible for the smooth appearance of the surface under the stereo microscope.

Despite considerable manipulation of the culture variables this tissue failed to produce any somatic embryos, (although some globular-stage embryoid structures were seen in tissue from Enkeim (see Plate 2.6)). Plate 2.5F represents the typical fate of such regions. This shows a structure very similar to the file-like meristems described earlier with its three distinct regions and the meristematic cells visible as a line of darker tissue situated below the tissue's surface. This it would seem therefore that the so called PE tissues might be just a variation on the formation of the domed meristematic regions, and that given time they would develop into such a structure. The large cells formed at the surface on these structures (see Plates 2.5C and 2.5F) would be responsible for the slightly roughened or cobbled texture seen under the stereo microscope after the tissue had lost its smooth appearance.

The reasons why the tissue identified optimistically as PE did not respond can only be speculated upon. To follow the ideas of the cells being locked into periclinal divisions, it is noticeable that all the cells in these structures had their longitudinal axis parallel to the tissue surface, and that they continued to divide in this way if maintained in the first stage medium. It is possible that once again these cells were by some means constrained by this pattern formation and thus would not be able to undertake the types of divisions necessary for embryogenesis to proceed.

Certainly the orientation of the meristematic cells at the surface of the PE tissue differed from that of the embryogenic tissues of cassava (Stamp, 1987); in these tissues the meristematic cells were orientated with their longitudinal axes perpendicular to the surface. Whether this might be some diagnostic feature of embryogenic tissue is not known but must be worth further investigation, because such a tool would be a more useful than macroscopic observations alone, for screening different tissues for their embryogenic competence. Such a system might have enabled the tissue in this study to have been identified as non-competent at an early stage and so prevented unnecessary efforts in the manipulation of the culture system on a tissue that was unlikely to be responsive.

Although no somatic embryos were ever recovered, the two structures illustrated in Plate 2.6 were seen in tissue of Enkeim which had been exposed to the first stage medium for 20 days; out

of numerous replicates examined, these structues were seen in this specimen only. There is, however, a possibility that incorrect planes of sectioning prevented identification in others.

Exactly what these meristematic structures are is not known but one interpretation is that they are globular embryos each possessing a distinct suspensor. Woodward (1989) and Hussey (1978) both report and provide illustrations of similar "somatic embryos". In these cases, as here, the suspensor was many cells thick - in A. thaliana about five - which contrasts with the file of single cells that act as the suspensor in zygotic embryos of A. thaliana. The globular structures on these suspensor-like structures are highly meristematic and are enclosed with a distinct protoderm, the cells of which have large nuclei with red nucleoli.

That these represent the early stages of somatic embryo formation could only be proved if they were shown to develop to the mature stages, the critical factor being the ability to proceed to the heart-shaped stage. No such morphogenesis was seen macroscopically or microscopically.

The reasons for this could be the low frequency of globular embryo formation, making it statistically unlikely that the mature products should have been observed, the fact that the globular structures are unable to develop further due to their inherent structure or that the culture medium was not inductive to their continued development. The final possibility implies that embryogenesis could still be achieved in this species if enough

such meristematic structures were generated to allow a proper investigation of the inductive conditions required for their development.

C O N C L U S I O N

CONCLUSION

Overall this study can be considered to have been at least partially successful with regard to its original aims. Somatic embryogenesis, a primary objective, was not achieved apart from the possible production of two globular embryoids. On the other hand an organogenic culture system was developed which was capable of inducing high frequency shoot and root formation from a range of explant sources and genotypes. Although both of these variables affected the organogenic competence the simplicity, rapidity, high frequency and the number of shoots recovered, especially from hypocotyl explants, surpassed all but a few of the previously reported regeneration systems. Further, this study examined more variables than any other to date, and it considerably expands our understanding of the morphogenic competence of this species in culture.

The results obtained and described in Chapter 1 provide a culture system which satisfies the criteria set down by Tran Than Van (1981) as requisites for a morphogenic system, if it is to act as an effective model for the investigation of the developmental controls behind these processes. These are, as listed above, a simple, rapid, synchronous system that occurs at reproducibly high frequencies. In addition, a recalcitrant tissue source with as similar a genome as possible to the responsive type(s) should be

available to facilitate an investigation into the genetic control of morphogenesis. In this case the genotype Coimbra with its poor caulogenic competence was ideally suited for this role.

With the availability of a culture system possessing all of these qualities, it was decided not to concentrate exclusively on manipulating the culture variables in order to maximise the response of the more recalcitrant tissues, but instead to exploit the differences in organogenic competence in order to address some of the developmental questions underlying de novo morphogenesis.

This took the form, in Chapter 1, of attempts to elucidate the states of competence and determination for organ formation by assessing the effects of varied exposure to the first and second stage media on the type and degree of organogenesis and was continued in Chapter 2, with an anatomical investigation of the histological developmental changes in the hypocotyl tissue during these periods. Through such a dual study it was hoped to correlate the structural development of the tissue with the acquisition of competence and determination for organ formation.

By manipulating its duration, it was possible to show that the time of exposure to the first stage medium, containing 10^{-6}M 2,4-D and 10^{-7}M BAP, was critical for inducing competence for a specific developmental fate on exposure to a second stage medium which was supplemented with 10^{-6}M BAP. It was found that the tissue had no morphogenic competence until it had been exposed to the 2,4-D/BAP medium for four days at which time transfer to the second stage induced very high frequency rhizogenesis. The state of rhizogenic competence was transient and declined rapidly after

four days at which time the tissue became caulogenically competent until day twelve. After twelve days competence for high frequency shoot formation was lost, to be replaced by a second period of rhizogenic competence (see Figures 9 and 12).

It was also shown that by fixing the first stage and varying the exposure to the second stage medium before transfer to a growth regulator-free medium, that the tissue appeared to have become determined for root formation in the first stage (see Figure 13). While roots emerged on direct transfer to the basal medium, the tissue was only became competent for caulogenesis on the first stage medium as an inductive exposure of at least 24 hours to the second stage was required before shoots could be regenerated.

Through the histological studies it was shown that these two morphogenic events took place from spatially different parts of the cultured tissue, rather than from cells which went through successive stages of rhizogenic and caulogenic competence. All of the shoots were derived from the calluses that developed at each cut end of the explant while high frequency rhizogenesis occurred from the highly organised meristematic tissue that developed from the stelar region of the original hypocotyl. The latter resembled pericyclic tissue (see Plate 2.3) and the roots were formed on transfer to the second stage from the slight bulges in its surface.

It is considered that these observations were consistent with the recorded rapidity of the rhizogenic event after transfer to the second stage and the lack of need for further induction by growth regulators as seen in Figure 13. Further it seems to substantiate the hypothesis that the tissue was determined for

rhizogenesis at the time of subculture to the second stage and that they were produced from already formed initials. This was borne out when the anatomy of these structures was studied (see Plate 2.3) and it is considered that the tissue of the central region need only perform a few coordinated periclinal and anticlinal divisions to become differentiated root primordia, with the removal of the 2,4-D possibly providing the permissive conditions for this to proceed.

The loss of competence for root formation between four and twelve days was correlated with a change in the structure of the tissue at this time and with the tissues period of maximum caulogenic competence (see Figure 9 and 12). From the results obtained in Chapter 1 and 2 it is not possible to come to any firm conclusions as to why the ability to regenerate roots is lost during this time. It could be that there is some antagonistic effect between the two types of organogenesis with competence for shoot formation in some way suppressing the ability to produce roots. Alternatively, the structural changes that occured in the central region after four days on the first stage medium in which the protodermal-like layer was lost to one with differentiated epidermal-like properties (see Plate 2.3I and 2.3J) might be important. The loss of the meristematic outer layer might prevent the coordinated divisions needed between it and layers below if a new root primodium is to be formed.

Clearly this is an area that needs further investigation if the reasons for the transient loss of rhizogenic competence are to be revealed. Culturing tissues for various times on the first

stage medium prior to transfer to the growth regulator-free medium should provide information as to the degree of rhizogenic competence of this tissue with increasing exposure to the 2,4-D/BAP medium and whether this, or the antaganistic effect of caulogenically competent, cells is responsible for the loss of root forming ability.

The histological investigation of the callus at the time of maximum caulogenic competence showed that, unlike the roots, there was no evidence that the shoot meristems were preformed after culture on the first stage medium. This, once again correlates with the results of Figure 13 in which an exposure to the second stage medium was required before shoot formation occurred.

Caulogenic competence was closely correlated with the presence of a thin layer of meristematic cells around the periphery of the callus tissue (see Plate 2.4A and 2.4B). This was observed after culture on the first stage medium for between six and ten days only, after which the meristematic cells became increasingly organised into relatively large, multi-layered structures in which the cells were arranged in orderly files as a result of divisions that appeared to be almost exclusively in the periclinal direction (see Plate 2.5). In addition the thin layer of meristematic cells was not seen in the callus tissue of the recalcitrant genotype Coimbra at any age (see Plate 2.4). It would appear, therefore, that these meristematic cells must be both present, and present in a specific form, if the tissue is to be competent to produce shoots on subculture to the inductive conditions of the second stage medium.

The decline of competence for shoot formation with continued time in culture has been attributed in this and other species to karyological, epigenetic and physiological changes in the tissue (see Introduction and Negrutiu, 1976; Halperin, 1986). In this case, however, the loss of competence was so rapid that karyological and epigenetic causes would seem unlikely and, while there may be a physiological effect of continued exposure to the 2,4-D/BAP which reduces the ability to respond to the inductive conditions of the second stage, this work has provided an additional possible explanation for the loss of regenerative capacity. This is that the structural state of the tissue at the time of exposure to the inductive conditions may be critical in establishing the competence to respond.

It is possible that the cells of the large meristematic regions seen in the tissues after about twelve days in the first stage may be locked into the periclinal divisions that form them. Thus although they may have been epigenetically and physiologically competent to respond they would be unable to undertake the coordinated periclinal and anticlinal divisions needed to form a new tunica structure. It is not difficult to envisage that the more loosely organised, narrow layer of meristematic cells present at the time of maximum competence for shoot formation would have fewer such constraints and thus they might be better able to enter the division patterns needed to form a new primordium.

It is considered that the recalitrance of the PE tissue to differentiate somatic embryos under the conditions tested, may also be due to the form of the meristematic regions (see Plate 2.5D to 2.5F) placing constraints on the possible division patterns and so preventing embryo formation.

It is possible therefore that a tissue must be in the physiologically and structurally correct states before it is competent for a specific morphogenic fate. If not in the correct physiological state then the chemical signal will not be effective at triggering the cells to divide in a specific pattern. However, structural effects may also be important in that the cells which have received, and are responsive to the signal, could be prevented from dividing in the necessary manner by the physical constraints placed upon them by the tissue of which they are a part.

Designing experimental systems that would allow the separation of the epigenetic, physiological and structural aspects of a tissue and their respective importance in imparting, or preventing, morphogenic competence would not be easy, but of great importance in increasing our understanding of the level at which plant development is controlled.

In order for organogenesis to proceed the 2,4-D had to be effectively removed from the medium. If this did not happen then the meristematic tissue developed to form increasingly multi-layered structures. Continued exposure to the 2,4-D thus prevented morphogenesis in this tissue as in many other reported cases (Murashige, 1974; Tisserat et al., 1979). Its removal, on the other hand, was associated with a change in the pattern of cell

division and was permissive for the organisation required to form new roots and shoots. As we have seen, in the case of the latter BAP was needed in the second stage if high frequency caulogenesis was to occur.

The role of the growth regulators in the second stage is not clear and can only be speculated on at this time. Williams and Maheswaran (1986) reported that BAP induced a change in the direction of cell divisions in tissue of Trifolium. Whether this was an active process with the cytokinin directly affecting the orientation of the cell-plate or whether removal of the 2,4-D (and increase in BAP in the case of shoot formation) acts permissively to just allow the cells to divide is not known. In the latter case the tissue would, respond to these conditions depending on its physiological and structural states (ie. its competence) at the time of exposure to such conditions. Such a hypothesis correlates with the ideas of Green (1980) and Linthilac (1984) (see Introduction) whereby the cells are influenced to divide by the cultural conditions, and these patterns of divisions, which are controlled by the physical forces acting on them, will automonously produce new meristems. In such a case therefore, the 2,4-D would be necessary to induce cell division and possibly determination, but morphologically constraining if it persists in the culture system.

The great advantage of working with Arabidopsis rather than some other species is that its mechanisms of gene expression promises to become increasingly understood in the near future. In addition numerous mutants are available for investigating specific

problems. These and the large number of wildtypes that have yet to be screened could be of considerable use in addressing the questions raised above, and contributing information as to whether the states of competence and determination are controlled at the gene level, as apparently in animals (Waddington, 1966), or whether mechanical or structural factors have a significant role in plants, as suggested by Green (1980) and Linthilac (1984).

The culture system designed here has left a number of questions unanswered as to the whether the morphogenic potential of this species has been fully uncovered. It is probable that the recalcitrant genotypes could have their caulogenic response improved by careful manipulation of the culture variables. This would be of use, especially for the recovery of plants from in vitro systems with the important genetic model Columbia, as well as providing information as to their differing requirements for caulogenesis, while expanding our understanding of the factors responsible for recalcitrance and the reasons for its specificity to some genotypes. It would be especially valuable if the genetic basis of this major problem could be established.

Finally, further attempts should be made to achieve medium to high frequency somatic embryogenesis in this species. Although this was not achieved here there were some indications, notably the presence of the two globular structures in callus of the genotype Enkiem, that this may be attainable (see Section 6 and Plates 2.6). This would be important as it would facilitate an investigation of the genetic control of somatic embryogenesis in comparison with that of organogenesis.

With regard to the developmental studies, it is considered that it is important to exploit the morphogenic system developed in this study to further our understanding of the nature of competence and determination in plants. The timings of these states has been established, but more work needs to be done to examine the changes that occur within the cells at the critical times when competence is gained or lost. Detailed histochemical and electron microscopy might provide information as to the changes that take place, especially in the nucleus in the protodermal cells of the central region around day four, or the differences in the meristematic cells in the terminal calluses at the time of caulogenic competence and after. This should address the relative importance of the epigenetic, physiological and structural aspects of organogenic competence.

REFERENCES

ACEDO, G.A. (1986). Regeneration of Arabidopsis thaliana In Vitro. Plant Cell Tissue and Organ Culture 6 109-114.

AITCHISON, P.A., MACLEOD, A.J. and YEOMAN, M.M. (1978). Growth Patterns in Tissue (Callus) Cultures. Ch.12 In Plant Tissue and Cell Culture. Biology Monographs Vol. II. Street, H.E. (Ed.). pp. 267-306. Blackwell Scientific Publications, Oxford.

ALONI, R. (1987). Differentiation of Vascular Tissues. Ann. Rev. Plant Physiol. 38 179-204.

ALY, M.A.M. and OWENS, L.D. (1987). A Simple System for Plant Cell Microinjection and Culture. Plant Cell, Tissue and Organ Culture 10 159-174.

AMMIRATO, P.V. (1977). Hormonal Control of Somatic Embryo Development from Cultured Cells of Caraway : Interactions of Abscisic Acid, Zeatin and Gibberellic Acid. Plant Physiol. 59 579-586.

AMMIRATO, P.V. (1983a). Embryogenesis. In Handbook of Plant Cell Culture Vol.1. Evans, D.A. et al. (Eds.) pp. 82-123. Macmillan, New York.

AMMIRATO, P.V. (1983b). The Regulation of Somatic Embryo Development in Plant Cell Culture : Suspension Culture Techiques and Hormone Requirements. Biotechnology __ 68-74.

AMMIRATO, P.V. (1985). Patterns of Development in Culture. In Tissue Culture in Forestry and Agriculture. Henke, R.R. et al (Eds.) pp. 9 - 29 Plenum Press, New York.

AMOS, J.A. and SCHOLL, R.L. (1978). Induction of Haploid Callus from Anthers of Four Species of Arabidopsis Z. Pflanzenphysiol. 90 33-43.

AN, G., WATSON, B.D., CHIANG, C.C. (1985). Transformation of Tobacco, Tomato, Potato, and Arabidopsis thaliana Using a Binary Ti Vector System. Plant Physiol. 81 301-305.

ANAND, R. (1966). Preliminary Studies on Callus Culture of A. thaliana. A.I.S. 3 15.

ARNISON, P.G. and BOLL, W.G. (1975). Isoenzymes in Cell Cultures of Bush Bean (Phaseolus vulgarus cv. Contender): Isoenzymatic Differences between Stock Suspension Cultures Derived from a Single Seedling. Con. J. Bot. 53 261-271.

BAJAJ, Y.P.S. (1983). In Vitro Production of Hapliods. In Handbook of Plant Cell Culture. Vol.1 Evans, D.A. et al. (Eds.) pp. 228-287. Macmillan, New York.

BANKS, M.S. (1979). Plant Regeneration from Cells from Two Growth Phases of English Ivy Hedera helix L. Z. Pflanzenphysiol. 92 349-353.

BAPAT, V.A. and RAO, P.S. (1984). Regulatory Factors for In Vitro Multiplication of Saddwood Tree (Santalum album Linn.) I. Shoot Bud Regeneration at Somatic Embryogenesis in Hypocotyl Cultures. Proc. Indian Acad. Sci. 93 19-27.

BAYLIS, M.W. (1980). Chromosomal Variation in Plant Tissues in Culture. Inter. Rev. of Cytol. Suppl. 11A 113-143.

BENNICI, A. (1974). Cytological Analysis of Roots, Shoots and Plants Regenerated from Suspension and Solid In Vitro Cultures of Hapliod Pelargonium. Z. Pflanzenphysiol. 72 199-205.

BINNS, A. and MEINS, J.F. (1973). Habituation of Tobacco Pith Cells for Factors Promoting Cell Division is Heritable and Potentially Reversible. Proc. Natl. Acad. Sci. U.S.A. 70 2660-2662.

BOWMAN, J.L., YANOFSKY, M.F. and MEYEROWITZ, E.M. (1988). Arabidopsis thaliana: A Review. In Miflin, B.F. (Ed.) Oxford Surveys of Plant Molecular and Cell Biology, Vol. 5 pp. 57-87.

BRETTELL, R.I.S., WERNICKE, W. and THOMAS, E. (1980). Embryogenesis from Cultured Immature Inflorescences of Sorghum bicolor. Protoplasma 104 141-148.

BROETJES, C. and KEEN, C. (1980). Adventitious Shoots : Do They Develop from One Cell. Euphytica 29 73-87.

BROWN, D.C.W. and THORPE, T.A. (1987). Plant Regeneration by Organogenesis. Ch.2 In Cell Culture of Somatic Cell Genetics of Plants Vol. 3. Plant Regeneration of Genetic Variability. Vasil. I.K. (Ed) pp Academic Press Inc., London.

BUI-DANG-HA (1974). Regeneration of Complete Plants of Asparagus from Protoplast Cultures. Abst. Nr. 261 The 3rd Inter. Cong. of Plant Tissue and Cell Culture. Leicester.

BUSH, S.R., EARLE, E.D. and LANGHANS, R.W. (1976). Plantlets from Petal Segments, Petal Epidermis and Shoot Tips of the Periclinal Chimera Chrysanthemum morifolium "Indianapolis". Am. J. Bot. 63 729-737.

CARMAN, J.G., JEFFERSON, N.E. and WILLIAM, F.C. (1987). Induction of Embryogenic Triticm aestivum L. Calli. I. Quantification of Genotype and Culture Media Effects. Plant Cell Tissue and Organ Culture 8 101-113.

CHEN, T.H.H., MAROWITCH, J. and THOMPSON, B.G. (1987). Genotypic Effects on Somatic Embryogenesis and Plant Regeneration from Callus Cultures of Alfalfa. Plant Cell Tissue Organ Culture 8 73-81.

CHRISTIANSON, M.L. (1985). An Embryogenic Culture of Soybean : Towards a Theary of Somatic Embryogenesis. In Tissue Culture in Agriculture and Forestry. Henke, R.R. et al. (Eds.). pp. 83-103. Plenum Press, New York.

CHRISTIANSON, M.L. (1987). Causal Events in Morphogenesis. In Plant Cell and Tissue Culture : Plant Biology Vol 3 Green, C.E. et al (Eds). pp. 45-55. Alan R. Liss Inc.,

CHRISTIANSON, M.L. and WARNICK, D.A. (1983). Competence and Determination in the Process of In Vitro Shoot Organogenesis. Develop. Biol. 95 288-293.

CHRISTIANSON, M.L. and WARNICK, D.A. (1984). Phenocritical Times in the Process of In Vitro Shoot Organogenesis. Develop. Biol. 101 382-390.

CHRISTIANSON, M.L. and WARNICK, D.A. (1985). Temporal Requirements for Phytohormone Balance in the Control of Organogenesis In Vitro. Develop. Biol. 112 494-497.

CHRISTIANSON, M.L. and WARNICK, D.A. (1988). Organogenesis In Vitro as a Developmental Process. Hort.science 23(3) 515-519.

COCKING, E.C. (1987). Plant Cell Biology in the 21st Century : The Needs of Plant Cell and Tissue Culture. In Plant Tissue and Cell Culture : Plant Biology Vol.3. Green, C.E. et al. (Eds.). pp. 3-15. Alan R. Liss Inc., New York.

COLEMAN, G.D. and ERNST, S.G. (1990). Shoot Induction and Competence in Callus Determination in Populus deltoides Plant Science 71 83-92.

CONN, H.J., DARROW, M.A. and EMMEL, V.M. (1965). Staining Procedures. Biological Stain Commission. Williams and Wilkins Company, Baltimore.

CONNER, S. (1989). Simple Weed Could Hold Key to Genetics. New Scientist 4th Feb. p. 38.

CORCOS, A. and LEWIS, R. (1971). Preliminary Report to Callus Formation and Growth in Vegetable-Juice and White Liquid Media. A.I.S. 8 9-10.

CORCOS, A., PIPER, B. and LEWIS, R. (1973). Redifferentiation on Normal Arabidopsis Plants from Callus Culture. A.S.I. 10 10.

D'AMATO, F. (1977). Cytogenetics of Differentiation in Tissue and Cell Cultures. In Applied and Fundamental Aspects of Plant, Cell, Tissue and Organ Culture, Reinert, J. and Bajaj, Y.P.S. (Eds.) pp. 343-357. Springer-Verlag, Berlin and New York.

DAMM, B. andd WILLMITZER, L. (1988). Regeneration Of Fertile Plants from Protoplasts of Different Arabidopsis thaliana Genotypes. Mol. Gen. Genet. 213 15-20.

DIETERT, M.F., BARRON, S.A. and YODER, D.C. (1982). Effects of Genotype and In Vitro Culture in the Genus Brassica Plant Sci. Letts. 26 233-240.

DURAND, J., POTRYKUS, I. and DONN, G. (1973). Plantes issues de Protoplastes de Petunia. Z. Planzenphysiol. 69 26-34.

ESAU, K. (1977). Anatomy of Seed Plants. 2nd. Edition John Wiley and Sons, New York.

EVANS, D.A. (1989). Techniques : Plant Cells at Tissue Culture Ch.3. In Plant Biotechnology. Kung, S-d and Amtzen, C.J. (Eds.) pp 53-76. Butterworths, Boston.

EVANS, D.A., SHARP, W.A. and FLICK, W.R. (1981). Growth and Behaviour of Cell Cultures : Embryogenesis and Organogenesis. In Plant Tissue Culture : Methods and Applications in Agriculture. Thorpe, T.A. (Ed.) pp. 44-114. Academic Press,

FEENSTRA, W.J. (1965). An Emasculation Technique. A.S.I. 2 34.

FELDMANN, K.A. and MARKS, M.D. (1986). Rapid and Efficient Regeneration of Plants from Explants of Arabidopsis thaliana. Plant Science 47 63-69.

FEUNG, C., MUMMA, R.O. and HAMILTON, R.H. (1974). Metabolism of 2,4-Dichlorophenoxyacetic Acid VII: Biological Properties of Amino Acid Conjugates. J. Agric. Food Chem. 22 3-7-309.

FEUNG, C., HAMILTON, R.N. and MUMMA, R.O. (1975). Metabolism of 2,4-Dichlorophenoxyacetic Acid VII. Biological Properties of Five Species of Plant Callus Tissue Cultures. J. Agric. Food Chem. 23 373-376.

FLAVELL, R. (1980). The Molecular Characterisation at Organisation of Plant Chromosomal DNA Sequences. Ann. Rev. Plant Physiol. 31 569-596.

FLICK, C.E., EVANS, D.A. and SHARP, W.R. (1983). Organogenesis. Ch.2 In Handbook of Plant Cell Culture. Vol. 1 Evans et at. (Eds.) pp. 31-81. Macmillan, New York.

FORD, K.G. (1990). Plant Regeneration from Arabidopsis thaliana Protoplasts. Plant Cell Reports 8 534-537.

FOSKET, D.E. (1980). Hormonal Control of Morphogenesis in Cultured Tissues. In Plant Growth Substances Skoog, F. (Ed.). pp. 362-369. Springer-Verlag, Berlin.

FURU YA, M. (1984). Cell Division Patterns in Multicellular Plants Am. Rev. Plant Physiol. 35 349-373.

GAMBORG, D.L., MILLER, R.A. and OSRMA, K. (1968). Nutrient Requirements of Suspension Cultures of Soybean Root Cells. Exp. Cell Res. 50 151-158.

GAMBORG, D.L. and SHYLUK, J.P. (1981). Nutrition, Media and Characteristics of Plant Cell and Tissue Cultures. In Plant Tissue Culture Methods and Applications in Agriculture. Thorpe, T.A. (Ed.) pp. 21-44

GAUTHERET, R.J. (1959). La Culture des Tissus Vegetaux. Maison et Cie, Paris.

GEORGE, E.F. andd SHERINGTON, P.D. (1984). Plant Propagation by Tissue Culture Handbook and Directory of Commercial Laboratories. Eastman Press, Reading, England.

GLEDDIE, S. (1989). Plant Regeneration from Cell Suspension Cultures of Arabidopsis thaliana Heynk. Plant Cell Reports 8 1-5.

GREEN, P.B. (1980). Organogenesis : A Biophysical View. Ann. Rev. Plant Physiol. 31 51-82.

GRESSHOFF, P.M. and DOY, C.H. (1972). Haploid A. thaliana Callus and Plants from Anther Culture. Aust. J. Biol. Sci. 25 259-264.

GRICA, M., KUBALAKOVA, M. and TESKOVA, E. (1987). Somatic Embryogenesis in Vicia faba. Plant Cell Tissue and Organ Culture 9 167-171.

GUIDERONI, E. and DEMARLEY, Y. (1988). Histology of Somatic Embryogenesis in Cultured Leaf Segments of Sugarcane Plantlets. Plant Cell Tissue and Organ Culture 14 71-88.

HACCIUS, B. (1978). Question of Unicellular Origin of Non-Zygotic Embryos in Callus Cultures. Phytomorphology 28 74-81.

HALPERIN, W. (1973). The Use of Cultured Tissue in Studying Developmental Problems. Can. J. Bot. 5(10) 1801-1806.

HALPERIN, W. (1986). Attainment and Retention of Morphogenetic Capacity In Vitro. Ch.1 In Cell Culture of Somatic Cell Genetics of Plants Vol.3. Plant Regeneration of Genetic Variability. Vasil, I.K. (Ed.) pp Academic Press, Inc., London.

HALPERIN, W. and WETHERELL, D.F. (1965). Ammonium Requirement for Embryogenesis In Vitro. Nature 205 519-520.

HAUGHN, G.W. and SOMMERVILLE, C.R. (1988). Genetic Control of Morphogenesis in Arabidopsis. Dev. Genet. 73-89.

HE, D.G., YANG, Y.M., BRETRAM, J. and SCOTT, K.J. (1990). Histological Development of the Regenerative Tissue Derived from Cultured Immature Embryos of Wheat (Triticum aestivum). Plant Science 68 103-111.

HEINZ, D.J. and MEE, G.W.P. (1971). Morphologic, Cytogenetic and Enzymatic Variation in Sacharum Species Hybrids and their Cell Suspension Cultures. Am. J. Bot. 56 450-456.

HENDERSON, J.H.M., PHILLS, B.R. and WHATLEY, B.T. (1984). Sweet Potato. Ch.11 In Handbook of Plant Cell Culture. Vol. 2. Sharp, W.R. et al (Eds.). pp. 302-326. Macmillan Pub. Co., New York.

HENSHAW, G.G. (1979). Plant Tissue Culture : Its Potential for Dissemination of Pathogen-Free Germplasm and Multiplication of Plant Materials. In Plant Health. Abbels, D.L. and King, J.E. (Eds.). Blackwell, Oxford.

HENSHAW, G.G., O'HARA, J.F. and WEBB, K.J. (1982). Morphogenetic Studies in Plant Tissue Culture. In Differentiation In Vitro. Yeoman, M.M. and Trueman, D.E.S. (Eds.) pp. 231-251. British Soc. for Cell Biol. Symp. 4 Cambridge Univ. Press, Cambridge.

HICKS, G.S. (1980). Patterns of Organ Development in Plant Tissue Culture and the Problems of Organ Determination. Bot. Review 46 1-23.

HOLDER, N. (1979). Positional Information and Pattern Formation in Plant Morphogenesis and a Mechanism for the Involvement of Plant Hormones. J. Theor. Biol. 77 195-212.

HUANG, BEI CHU (1985). Morphogenesis in Arabidopius thaliana PhD Thesis University of Edinburgh, Edinburgh.

HUANG, BEI CHU and YEOMAN, M.M. (1984). Callus Proliferation and Morphogenesis in Tissue Cultures of Arabidopsis thaliana L.. Plant Sci. Letts. 33 353-363.

HUGHES, K.W. (1981). In Vitro Ecology: Exogenous Factors Affecting Growth and Morephogenesis in Plant Culture System. Env. and Expt. Botany 21 281-288.

HUSSEY, G. (1978). The Application of Tissue Culture to the Vegetable Propagation of Plants. Sci. Prog., Oxford 65 185-208.

JACOBSEN, H-J. (1981). Soluble Auxin Binding Proteins in Pea. Cell Biol Int. Rep. 5 768.

JARRET, R.L., SALAZER, S. and FERNANDEZ, R. (1984). Somatic Embryogenesis in Sweet Potato. Hort Science 19 397-398.

JONES, L.H. (1974). Factors Influencing Embryogenesis in Carrot Cultures (Daucus carota). Ann. Bot. 38 1077-1088.

KAMADA, H. and HARADA, H. (1979). Studies on Organogenesis in Carrot Tissue Cultures. II Effects of Amino Acids and Inorganic Nitrogenous Compounds on Somatic Embryogenesis. Z.Pflanzenphysiol. 91 453-463.

KAMADA, H. and HARADA, H. (1983). Studies on Nitrogen Metabolism During Somatic Embryogenesis in Carrot. Proc. 5th Inter. Cong. Plant Tissue and Cell Culture. PLANT TISSUE CULTURE 1982. 115-116.

KAMADA, H. and HARADA, H. (1984). Changes in the Endogenous Amino Acid Composition During Somatic Embryogenesis in Daucus carota. Plant and Cell Physiol. 25 27-38.

KAUL, V., MILLER, R.M., HUTCHISON, J.F. and RICHARDS, D. (1990). Shoot Regeneration from Stem and Leaf Explants of Dendrathanthema grandiflora Tzlelev. (syn. Chrysanthemum marifolium). Plant Tissue and Organ Culture 21 21-30.

KEATHLEY, D.E. and SCHOLL, R.L. (1983). Chromosomal Heterogeneity of A. thaliana Anther Callus and Regenerated Shoots and Plants. Z.Pflanzenphysiol. 112 247-255.

KOHLENBACH, H.W. (1977). Basic Aspects of Differentiation and Plant Regeneration from Cells and Tissue Cultures. In Plant Tissue Culture and its Biotechnical Application. Barz, W. (Ed.) pp. 355-364. Springer-Verlag, Berlin.

KOHLENBACH, H.W. (1978). Comparative Somatic Embryogenesis. Ch. 6 In Frontiers of Plant Tissue Culture. Thorpe, T.A. (Ed.) pp. 59-66. Int. Ass. for Plant Tissue Culture, Calgory.

KONAR, R.N. and NATURAJA, K. (1965). Experimental Studies in Rannunculus scleratus L. Development of Embryos from the Stem Epidermis. Phytomorphology 115 132-137.

KONAR, R.N., THOMAS, E. and STREET, H.E. (1972). Origin and Structure of Embryiods Arising from Epidermal Cells of the Stem of Rannunculua sceleratus. J. Cell Sci. 11 77-93.

KUMER, A and COCKING, E.C. (1986). Protoplast Fusion : A Novel Approach to Organelle Genetics in Higher Plants. Am. J. Bot.

LAIBACH, F. (1943). Arabidopsis taliana (L.) Objekt fur Genetische und Entwicklungsphysioloische Untersu chungen. Bot. Archiv. 44 439-455.

LANG, H. and KOHLENBACH, H.W. (1975). Morphogenese in Kulturen Isolierte Mesophyllzellen von Macleya cordata. In Form, Structure and Function of Plants. Johri, B.M. (Ed.) Commemoration Vol. Meerut, India.

LANGRIDGE, J. (1955). Biochemical Mutations in the Crucifer Arabidopsis thaliana (L.) Heynh.. Nature 176 260-261.

LEOWENBERG, J.R. (1965). Callus Culture of Arabidopsis. A.I.S. 2 34.

LINTILHAC, P.M. (1974). Differentiation, Organogenesis, at the Tectonics of Cell Wall Orientation. II Separation of Stress in a Two-Dimensional Model. Amer. J. Bot. 61(2) 135-140.

LINTILHAC, P.M. (1984). Positional Controls in Meristem Development : A Caveat and an Alternative. In Positional Controls in Plant Development. Barlow, P.W. and Carr, D.J. (Eds.). pp. 83-105. Cambridge Univ. Press, Cambridge.

LITZ, R.E. (1985). Somatic Embryogenesis in Tropical Fruit Trees. In Tissue Culture in Forestry and Agriculture. Henke, R.R. et al (Eds.) pp. 1789-193 Plenum Press, New York.

LU, C-Y. and VASIL, I.K. (1982). Somatic Embryogenesis and Plant regeneration in Tissue Cultures of Panicum maximum Jacq. Am. J. Bot. 69 77-81.

LUTZ, A. (1971). Adaptidudes Morphgenetiques des Cultures des Tissus Origne Unicellular. In Les Cultures de Tissus de Plantes. pp. 163-168. Editions de Centre Nat. Rech. Scient., Paris.

LUTZ, J.D., WONG, J.R., ROWE, J. TRICOLI, D.M. and LAWRENCE, R.H. (1985). Somatic Embryogenesis for Mass Cloning of Crop Plants In Tissue Culture in Forestry and Agriculture. Henke, R.R. et al (Eds.) pp 105-116. Plenun Press, New York.

LYNDON, R.F. (1990). Plant Development : The Cellular Basis : Topics in Plant Biology 3. Unwin Hyman, Boston.

MAEDA, E. and THORPE, T.A. (1979). Shoot Histogenesiss in Tobacco Callus Cultures. In Vitro 15(6) 415-424.

MAHESWARAN, G. and WILLIAMS, E.G. (1985). Origin and Development of Somatic Embryos Formed Directly on Immature Embryos of Trifolium repens In Vitro, Ann. Bot. 56 619-630.

McWILLIAM, A.A., SMITH, S.M. and STREET, H.E. (1974). The Origin and Development of Embryoids in Suspension Cultures of Carrot (Daucus carota). Ann. Bot. 38 243-250.

MEHRA, A. and MEHRA, P.N. (1974). Organogenesis and Plantlet Formation In Vitro in Almond. Bot. Gaz. 135 61-73.

MEIJER, E.G.M. and BROWN, D.C.W. (1987). Role of Endogenous Reduced Nitrogen and Sucrose in Rapid High Frequency Somatic Embryogenesis in Medicago sativa. Plant Cell Organ Tissue Culture 10 11-19.

MEINHERDT, H. (1984). Models of Pattern Formation at their Application to Plant Development. Chl In Positional Controls : Plant Development. Barlow, P.W. and Carr, D.J. (Eds,) pp 1-31 Cambridge Univ. Press, Cambridge.

MEINS, F. (1986). Determination and Morphogenetic Competence in Plant Tissue Culture. In Plant Cell Culture Technology. Yeoman, M.M. (Ed.). pp. 7-25. Blackwell, New York.

MEINS, J.F. and BINNS, A.N. (1979). Cell Determination in Plant Development. Bio. Sci. 29 221-225.

MEINS, J.F. and LUTZ, J. (1979). Tissue-Specific Variation in the Cytokinin Habituation of Cultured Tobacco Cells. Differentiation 15 1-6.

MEYEROWITZ, E.M. (1987). Arabidopsis thaliana. Ann. Rev. Gen. 21 93-111.

MEYEROWITZ, E.M. and PRUITT, R.E. (1985). Arabidopsis thaliana and Plant Molecular Genetics. Science 229 1214-1218.

MONTAGUE, M.J., ENNS, R.K., SIEGAL, N.R. and JAWORSKI, E.G. (1981). A Comparison of 2,4-dichlorophenoxyacetic Acidd Metabolism in Cultured Soybean Cells and in Embryogenic Carrot Cellss. Plant Physiol. 67 603-607.

MOK, M.C., MOK, D.W.S., DIXON, S.C., ARMSTRONG, D.J. and SHAW, G. (1982). Cytokinin Structure - Activity Relationships and the Metabolism of N^6 (Isopentenyl) Adenosine-8-^{14}C in Phaseolus Callus Tissues. Plant Physiol. 70 173-178.

MURASHIGE, T. (1974). Plant Propagation through Tissue Cultures. Ann. Rev. Plant Physiol. 25 135-166.

MURASHIGE, T. and HUANG, L-C. (1985). Organogenesis In Vitro : Structural, Physiological and Biochemical Aspects. In Biotechnology in International Agricultural Research. proc. of the Inter-Centre Seminar on International Agricultural Research April 1984. pp. 227-239 International Rice Research Institute, Manila, Philipines.

MURASHIGE, T. and NAKANO, R. (1967). Chromosome Complement as a Determinant of the Morphogenic Potential of Tobacco Cells. Am. J. Bot. 54 963-970.

MURASHIGE, T. and SKOOG, F. (1962). A Revised Medium for Rapid Growth and Boiassays within Tobacco Cultures. Physiol. Plant. 15 473-497.

NARASHIMHULU, S.B. and CHOPRA, V.L. (1988). Species Specific Shoot Regeneration Response of Cotyledonary Explants of Brassicas. Plant Cell Reports 7 104-106.

NARAYANASWAMY, S. (1977). Regeneration of Plants from Tissue Cultures. Ch.10 In Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Reinert, J. and Bajaj, Y.P.S. (Eds.) pp. 179-248. Springer-Verlag, Berlin and New York.

NEGRUTIU, I., BEEFFTINK, F. and JACOBS, M. (1975). A. thaliana as a Model System in Somatic Cell Genetics. Plant Sci. Letts. 5 293-304.

NEGRUTIU, I. (1976). In Vitro Morphogenesis in A. thaliana. A.I.S. 13 180-187.

NEGRUTIU, I., JACOBS, M. and CACHITA, D. (1978a). Some Factors Controlling In Vitro Morphogenesis of Arabidopsis thaliana. Z.Pflanzenphysiol. 86 113-124.

NEGRUTIU, I., JACOBS, M. and DeGEEF, W. (1978b). In Vitro Morphogenesis of Arabidopsis thaliana : The Origin of the Explant. Z.Pflanzenphysiol. 90 363-372.

NEGRUTIU, I. and JACOBS, M. (1978c). Factors which Enhance In Vitro Morphogenesis of A. thaliana. Z.Pflanzenphysiol. 90 423-430.

NISHI, T. and MITSUOKA, S. (1969). Occurance of Various Pliody Plants from Anther and Ovary Culture of Rice Plants. Jpn. J. Genet. 44 341-346.

NITSCH, J.P. (1967). Towards a Biochemistry of Flowering and Fruiting : Contributions of the "in vitro" Technique. Proc. of the XVII International Hort. Congresss. Vol. III.

O'BRIEN, T.P. and McCULLY, M.E. (1981). The Study of Plant Structure : Principles and Selected Methods. Termarcarphi PTY. Ltd., Melbourne, Australia.

PADMANABHAN, V., PADDOCK, E.F. and SHARP, W.R. (1974). Hormonal Control of Organogenesis in Lycopersicum esculantum Leaf Callus. Abst. Nr. 66 The 3rd Inter. Cong. of Plant Tissue and Cell Culture. Leicester.

PARKER, R.E. (1979). Introductory Statistics for Biology 2nd Ed. Edward Arnold, London.

PATTON, D.A. and MEINKE, D.W. (1988). High Frequency Plant Regeneration from Cultured Cotyledons of Arabidopsis thaliana. Plant Cell Reports 7 233-237.

PELISSER, B., BOUCHEFRA, O., PEPIN, R. and FREYSSINET, G. (1990). Production of Isolated Somatic Embryos from Thin Layers of Hypocotyls. Plant Cell Reports 9 47-50.

RAFF, J.W., HUTCHISON, J.F., KNOX, R.B. and CLARKE, A.E. (1979). Cell Recognition : Antigenic Determinents of Plant Organs and their Cultured Cells. Differentiation 12 179-186.

REDEI, G.P. (1975). Arabipopsis as a Genetic Tool. Ann. Rev. Genetics 9 111-127.

REINERT, J. (1958). Morphogenese und ihre Kontrolle an Gewebekulturen aux Carotten. Naturwissensschten 45 344-345.

REINERT, J. (1959). Uber die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewegekulturen aus Karotten. Planta(Berl.) 34 657-669.

REINERT, J., TAZAWA, M. and SEMENOFF, S. (1967). Nitrogen Compounds as Factors of Embryogenesis In Vitro. Nature 216 1215-1216.

REYNOLDS, T.L. (1989). Adventive Organogenesis from Somatic Tissue Cultures of Solanum carolinense : Origin and Development of Regenerated Plants. Am. J. Bot. 76(4) 609-613.

ROSS, M.K. and THORPE, T.A. (1973). Physiological Gradients and Shoot Initiation in Tobacco Callus Cultures. Plant Cell Physiol. 14 473-480.

SACHS, T. (1978). Patterned Differentiation in Plants. Differentiation 11 65-73.

SACRISTAN, M.D. and MELCHERS, G. (1969). The Caryological Analysis of Plants Regenerated from Tumerous and other Callus Cultures of Tobacco. Mol. Gen. Genet. 105 317-333.

SCHOLL, R.L. and AMOS, J.A. (1980). Isolation of Double-Haploid Plants Through Anther Culture in A. thaliana. Z.Pflanzenphysiol. 90 407-414.

SCHOLL, R.L., KEATHLEY, D.E. andd BARIBAULT, T.J. (1981). Enhancement of Root Formation and Fertility in Shoots Regenerated from Anther and Seedling Derived Callus Cultures of A. thaliana. Z.Pflanzenphysiol. 104 225-231.

SCHOLTEN, H.J. and FEENSTRA, W.J. (1985). Embryo-like Structures in Anther Culture of Arabidopsis thaliana. A.I.S. 21 65-68.

SENGUPTA, C. and RAGHAVAN, V. (1979). Somatic Embryogenesis in Current Cell Suspension. I. Pattern of Protein and Nucleic Acid Synthesis. J. Expt. Bot. 31 247-258.

SHARMA, K.K. and BOJWANI, S.S (1990). Histological Aspects of In Vitro Root and Shoot Differentiation from Cotyledon Explants of Brassica junca (L.). Plant Science 69 207-214.

SHARP, W.R., EVANS, D.A. and SONDAHL, M.R. (1982). Application of Somatic Embryogenesis to Crop Improvements. In PLANT TISSUE CULTURE 1982 Proc. 5th Inter. Cong. Plant Tissue and Cell Culture. 759-762.

SHARP, W.R., SONDDAHL, M.R., CALDAS, L.S. and MARAFFA, S.B. (1980). The Physiology of In Vitro Asexual Embryogenesis. Hort. Review 2 268-310.

SHEN-MILLAR, J. and SHARP, W.R. (1966). An Improved Medium for Rapid Initiation of Arabidopsis Tissue Culture from Seed. Bull. Torrey Bot. Club 93 68-75.

SHEPARD, J.F. (1980). Abscisic Acid Enhanced Shoot Formation in Protoplast-Derived Calli of Potato Plant Sci. Letts. 18 327-333.

SHININGER, T.L. (1979). The Control of Vascular Development. Ann. Rev. Plant Physiol. 30 313-337.

SHOEMAKER, R.C., COUCHE, L.C. and GALBRAITH, D.C. (1986). Characterisation of Somatic Embryogenesis and Plant Regeneration in Cotton (Gossipium hirsutum). Plant Cell Reports 3 178-181.

SKOOG, F. (1971). Aspects of Growth Factor Interactions in Morphogenesis of Tobacco Tissue Cultures. In Les Cultures de Tissus de Plantes. Colloquess Inter. C.N.R.S. Vol. 193.

SKOOG, F. and MILLER, C.O. (1957). Chemical Regulation of Growth and Organ Formation in Plant Tissues Cultured In Vitro. Symp. Soc. Exp. Biol. 11 118-131.

SMITH, M.K. and DREW, R.A. (1990). Current Applications of Tissue Culture in Plant Propagation and Improvement. Aust. J. Plant Physiol, 17 267-289.

SMITH, D.R. and THORPE, T.A. (1975). Root Initiation in Cuttings of Pinus radiata Seedlings. I. Developmental Sequence. J. of Expt. Bot. 26 184-192.

SMITH, S.M. and STREET, H.E. (1974). The Decline of Embryogenic Potential as Callus and Suspension Cultures of Carrot (Daucus carota L.) are Serially Subcultured. Ann. Bot. 38 223-241.

STAMP, J.A. (1987). Somatic Embryogenesis in Cassava : The Anatomy and Morphology of the Regeneration Process. Ann. of Bot. 59 451-459.

STERLING, C. (1951). Origin of Buds in Tobacco Stem Segments Cultured In Vitro. Am. J. Bot. 38 761-767.

STEWARD, F.C. (1970). Totipotency, Variation and Clonal Development of Cultured Cells. Endeavor 29 117-124.

STEWARD, F.C., MAPES, M.O. and MEARS, K. (1958). Growth and Organised Development of Cultured Cells. II. Organisation in Callus Grown from Freely Suspended Cells. Amer. J. Bot. 45 705-708.

STREET, H.E. (1976). Experimental Embryogenesis - The Totipotency of Cultured Plant Cells. In The Developmental Biology of Plant and Animals. Graham C.F. and Wareing P.F. (Eds.) pp 73-90. Blackwell, Oxford.

STREET, H.E. (1979). Embryogenesis and Chemically Induced Organogenessis. In Plant Cell and Tissue Culture. Sharp, W.R. et al. (Eds.). pp. 127-153. State Univ. Press, Columbus, Ohia.

STOUTMEYER, V.T. and BRITT, O.K. (1965). The Behavoir of Tissue Cultures of English and Algerian Ivy of Different Growth Phases. Am. J. Bot. 52 805-810.

STUART, D.A., NELSEN, J., STRICKLAND, S.G. and NICHOL, J.W. (1985). Factors Affecting Developmental Processes in Alfalfa Cell Cultures In Tissue Culture in Forestry and Agriculture Henke, R.R. et al (Eds.) pp 59-73. Plenum Press, New York.

STYER, D.J. (1985). Bioreactor Technology for Plant Propagation. In Tissues Culture in Forestry and Agriculture. Henke R.R. et al. (Eds.) pp. 117-130. Plenum Press, New York.

SUSSEX, I.M. (1982). Determination of Plant Organs and Cells. In Genetic Engineering in Plants : An Agricultural Perspective. Kosge, T. et al. (Eds.). pp 443-451. Plenum Press, New York.

THEOLOGISS, A. (1989). Auxin-Regulated Gene Expression in Plants. Ch.10 In Plant Biotechnology. Kung, S-d and Aratzen, C.J. (Eds.) pp 229-243 Butterworths, Barton.

THOMAS, E., HOFFMAN, F. POTRYKUS, I. and WERNZEL, G. (1976). Protoplast Regeneration and Stem Embryogenesis of Hapliod Androgenic Rape. Molec. Gen. Genet. 145 245-247.

THOMAS, E. and STREET, H.E. (1970). Organogenesis in Cell Suspension Cultures of Atropa bellandonna L. and Atropa belladonna cultivar Lutea Doll. Ann. Bot. 34 657-669.

THOMAS, E. and WERNICKE (1978). Morphogenesis in Herbaceous Crop Plants. Ch. 41 In Frontiers of Plant Tissue Culture. I.P.T.C. 1978. Thorpe, T.A. (Ed.) pp. 403-410. Int. Ass. Plant Tissue Culture, Calgary.

THORPE, T.A. (1978). Physiological and Biochemical Aspects of Organogenesis In Vitro. Ch.5 In Frontiers of Plant Tissue Culture 1978. Thorpe, T.A. (Ed.). pp. Univ. of Calgary, Calgary, Canada.

THORPE, T.A. (1980). Organogenesis In Vitro : Structural, Physiological and Biochemical Aspects. Int. Rev. of Cytol. Suppl. 11A 71-111.

THORPE, T.A. (1982). Physiological and Biochemical Aspects of Organogenesis In Vitro. Plant Tissue and Cell Culture Proc. 5th Int. Cong. 121-124.

THORPE, T.A. and MEIER, D.D. (1973). Effects of Gibberellic Acid and Abscissic Acid on Shoot Formation in Tobacco Callus Cultures. Physiol. Plant. 29 121-124.

THORPE, T.A. and MURASHIGE, T. (1968). Starch Accumulation in Shoot forming Tobacco Callus Cultures. Science 160 421-422.

THORPE, T.A. and MURASHIGE, T. (1970). Some Histological Changes Underlying Shoot Initiation in Tobacco Callus Cultures. Can. J. Bot. 48 277-285.

TISSERAT, B., ESAN, E.B. and MURASHIGE, T. (1979). Somatic Embryogenesis in Angiosperms. Ch.1 In Hort. Reviews. Vol.1 Janick, J. (Ed.) AVI Publishing Co., Westport, Conneticut.

TORREY, J.G. (1967). Morphogenesis in Relation to Chromosomal Constituants in Long-Term Plant Tissue Cultures. Physiol. Plant. 20 265-275.

TRAN THANH VAN, K.M. (1978). Morphogenesis in The Thin Cell Layers. In Frontiers of Plant Tissue Culture 1978 Thorpe, T.A. (Ed) Category: Int. Assoc. Plant Tissue Culture pp 556.

TRAN THANH VAN, K.M., (1981a). Control of Morphogenesis In Vitro Cultures. Ann. Rev. Plant Physiol. 32 291-311.

TRAN THAN VAN, K. (1981b). Control of Morphogenesis by Inherent and Endogenously Applied Factors in Thin Cell Layers. Int. Rev. of Cytol. Suppl. 11A 175-194.

VASIL, I.K. (1982). Somatic Embryogenesis and Plant Regeneration in Cereals and Grasses. Proc. 5th Inter. Cong. Plant Tissue and Cell Culture PLANT TISSUE CULTURE 1982.

VASIL, I.K. (1985). Somatic Embryogenesis and its Consequences in the Gramineae. In Tissue Culture in Forestry and Agriculture. Henke, R.R. et at. (Eds.) pp. 31-47. Plenum Press, New York.

VASIL, I.K. and VASIL, V. (1980). Clonal Propagation Int. Rev. Cytol. Suppl. 11A 145-173.

VASIL, V. and VASIL, I.K. (1985). Histology of Somatic Embryogenesis in Cultured Immature Embryos of Maize (Zea mays L.). Protoplasma 127 1-8.

VILAPLANA, M. and MULLINS, G. (1989). Regeneration of Grapevines (Vitis spp.) In Vitro : Formation of Adventitious Buds in Hyptcotyls and Cotyledons of Somatic Embryos. J. Plant Physiol. 134 413-419.

WADDINGTON, C.H. (1966). Principles of Development and Differentiation. MacMillan, New York.

WARREN WILSON, J. and P.M. (1984). Control of Tissue Patterns in Normal Development and in Regeneration. In Positional Controls in Plant Development. Barlow, P.W. and Carr, D.J. (Eds.). pp. 238-280. Cambridge Univ. Press, Cambridge.

WANG, L., HUANG, B., HE, M. and HAO, S. (1990). Somatic Embryogenesis and its Hormonal Regulation in Tissue Cultures of Freesia refracta. Ann. Bot. 65 271-276.

WENTMORE, R.H. and REIR, J.P. (1963). Experimental Induction of Vascular Tissues in Callus of Angiosperms. Am. J. Bot. 50 418-430.

WETHERELL, D.F. (1977). In Vitro Embryiod Formation in Cells Derived from Somatic Plant Tissues. In Propagation of Higher Plants through Tissue Culture. Hughes et al. (Ed.). pp.102-124. Technical Information Centre U.S. Dept. of Energy.

WETHERELL, D.F. and DOUGALL, D.K. (1976). Sources of Nitrogen Supporting Growth and Differentiation in Vascular Plants. Am. J. Bot. 50 418-430.

WILLIAMS, E.G. and MAHESWARAN, G. (1986). Somatic Embryogenesis : Factors Influencing Coordinating Behaviour of Cells as an Embryogenic Group. Ann. of Bot. 57 443-462.

WOLPERT, L. (1971). Positional Information and Pattern Formation. Current Topics in Develop. Biol. 6 183-224.

WOODWARD, B.R. (1989). Somatic Embryogenesis in Maize (Zea mays L.). MSc Thesis Univ. of Witwatersnud, Johanasberg.

YAMAGUCHI, T. and NAKAJIMA, T. (1974). Effect of Abscisic Acid on Adventitious Bud Formation in Cultured Tissue Derived both from Root Tubers of Sweet Potato and from Stem Tubers of Potato. Absr. Nr. 65 The 3rd Inter. Cong. of Plant Tissue and Cell Culture. Leicester.

YEOMAN, M.M. (1970). Early Events in Callus Cultures. Inter. Rev. of Cytol. 29 383-409.

YEOMAN, M.M. and FORCHE, E. (1980). Cell Proliferation and Growth in Callus Cultures. Inter. Rev. of Cytology Suppl. 11A 1-24.

YEOMAN, M.M. and BROWN, R. (1971). Effects of Mechanical Stress in the Phase of Cell Division in Developing Callus Cultures. Ann. Bot. 35 1101-1112.

YEUNG, E.C., AITKEN, J., BIONDI, S. and THORPE, T.A. (1981). Shoot Histogenesis in Cotyledon Explants of Radiata Pine. Bot. Gaz. 142(2) 494-480.

YOKAYAMA, K. and JONES, W.H. (1965). Abstract. Plant. Physiol. 40 Suppl. p.1 .

ZHANG, H. and SOMERVILLE, C.R. (1987). Transfer of Maize Transposable Element Mui into Arabidopsis thaliana. Plant Science 48 165-173.

ZEIBUR, N.K. (1965). Tissue Culture. A.I.S. 2 34-35.

APPENDIX

APPENDIX 1 Chemicals Employed and their Respective Sources

Appendix 1.1 Chemicals Utilised in Chapter 1

Product	Source
agar	Oxoid Ltd., Basingstoke, England
Ammonium sulphate	BDH Chemicals Ltd, Poole, England
Analar sucrose	Fisons plc., Loughborough England
Casein hydrolosate	Sigma Chemical Co. Ltd, Poole, England
Charcoal activated	Sigma Chemical Co. Ltd,
2,4D, BAP, 1AA, NAA kinetin and zeatin	Sigma Chemical Co. Ltd,
MS basal medium	Flow Laboratories, Irvine, Scotland
Parafilm	American Can Company, Greenwich, U.S.A.
Petri dishes	Sterelin Ltd., Teddington, England
Sodium hypochlorite	BDH Chemicals Ltd.
Tween 80	BDH Chemicals Ltd.

APPENDIX 2 Mean Widths (mm) with Standard Errors for Hypocotyl Tissue of Various Genotypes Cultured on First Stage Medium

Time in the First Stage Medium (days)

Genotype	4	6	8	10	12	14	16	18	20
Co.	0.34 ±0.008	0.58 ±0.02	1.06 ±0.02	1.43 ±0.03	1.78 ±0.03	2.15 ±0.02	2.78 ±0.04	3.01 ±0.06	3.92 ±0.11
Bla.	0.36 ±0.009	0.53 ±0.01	0.93 ±0.02	1.39 ±0.02	1.77 ±0.04	2.08 ±0.03	2.41 ±0.04	2.76 ±0.04	3.39 ±0.06
Col.	0.34 ±0.08	0.44 ±0.09	0.61 ±0.03	0.92 ±0.04	0.88 ±0.04	1.10 ±0.06	1.22 ±0.07	1.99 ±0.1	2.23 ±0.14
Est.	0.35 ±0.009	0.59 ±0.03	0.95 ±0.03	1.34 ±0.04	1.71 ±0.09	2.14 ±0.06	2.35 ±0.06	2.95 ±0.11	4.23 ±0.27
Chi.	0.33 ±0.008	0.47 ±0.01	0.81 ±0.03	1.16 ±0.03	1.28 ±0.04	1.76 ±0.04	1.96 ±0.04	2.42 ±0.06	2.85 ±0.05
En.	0.35 ±0.009	0.61 ±0.009	1.07 ±0.02	1.50 ±0.03	1.84 ±0.03	2.3 ±0.03	2.96 ±0.06	3.41 ±0.06	3.74 ±0.13
Di.	0.31 ±0.005	0.54 ±0.02	1.06 ±0.01	1.38 ±0.02	2.24 ±0.05	2.9 ±0.05	4.23 ±0.09	5.30 ±0.18	5.62 ±0.14
Be.	0.28 ±0.007	0.52 ±0.02	0.97 ±0.03	1.02 ±0.03	1.51 ±0.03	1.91 ±0.04	2.23 ±0.06	2.52 ±0.01	2.82 ±0.09
Lad.	0.30 ±0.007	0.60 ±0.01	1.21 ±0.03	1.50 ±0.01	1.87 ±0.04	2.49 ±0.07	3.01 ±0.07	4.73 ±0.11	5.12 ±0.11

APPENDIX 3 Mean Number of Shoots Produced Per Responding Cells from Nine Genotypes Exposed to Varying First Stage Durations at 21 Days on the Second Stage Medium

Time in the First Stage Medium (days)

Genotype	6	8	10	12	14
Col.	1.41 ±0.15	1.78 ±0.22	1.79 ±0.14	1.00	-
Co.	1.13 ±0.10	1.13 ±0.10	1.20 ±0.12	-	-
En.	12.09 ±0.40	12.05 ±0.63	11.57 ±0.42	3.40 ±0.25	1.71 ±0.28
Be.	9.52 ±0.82	6.85 ±0.54	6.70 ±0.92	2.53 ±0.40	-
Chi.	10.19 ±0.40	13.40 ±1.12	8.52 ±0.32	4.10 ±0.64	3.20 ±0.45
Lad.	7.43 ±0.58	7.09 ±0.59	7.36 ±0.94	4.07 ±0.63	2.63 ±0.49
Di.	3.20 ±0.39	5.50 ±0.98	8.57 ±0.42	3.47 ±0.50	2.56 ±0.60
Bla.	5.20 ±0.25	9.33 ±0.64	8.81 ±0.59	2.87 ±0.40	2.00 ±0.62
Est.	6.40 ±0.58	10.48 ±0.39	10.29 ±0.77	4.06 ±0.62	3.62 ±0.41